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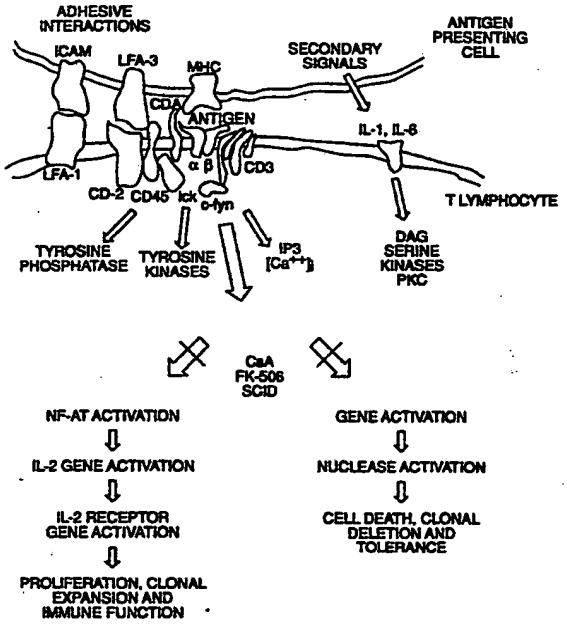
(54) Title: SCREENING METHODS FOR IMMUNOSUPPRESSIVE AGENTS

(57) Abstract

Activation of NF-AT-dependent transcription, including agents which interfere with the production, modification of the nuclear or cytoplasmic subunits, or the nuclear import of the cytoplasmic subunits. In particular, screening tests for novel immunosuppressants are provided based upon the ability of NF-AT to activate transcription.



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SCREENING METHODS FOR IMMUNOSUPPRESSIVE AGENTS

This invention was made in the course of work supported by the U.S. Government, which has certain rights in this invention.

BACKGROUND OF THE INVENTION

The immune response is coordinated by the actions of cytokines produced from activated T lymphocytes. T lymphocytes having a broad spectrum of antigen receptors are produced in the thymus as a product of the processes of selection and differentiation. When these T cells migrate to the peripheral lymphoid organs and encounter antigen, they undergo activation, during the process of which they produce large numbers of cytokines that act upon other cells of the immune system to coordinate their behavior to bring about an effective immune response.

T lymphocyte activation involves the specific regulation of many genes from minutes after the antigen encounter until at least 10 days later. T cells may also be activated by stimuli such as the combination of a calcium ionophore (e.g., ionomycin) and an activator of protein kinase C, such as phorbol myristate acetate (PMA). Several lectins, including phytohemagglutinin (PHA) may also be used to activate T cells (Nowell, P.C. (1990) Cancer Res. 20:462-466). The T cell activation genes are roughly grouped based on the time after stimulation at which each gene is regulated. Early genes trigger the regulation of subsequent genes in the activation pathway.

Because of the critical role of the T lymphocyte, agents that interfere with the early activation genes, such as cyclosporin A and FK506, are effective immunosuppressants. These early activation genes are regulated by transcription factors, such as NF-AT, that in turn are regulated through interactions with the antigen receptor. These transcription factors act through enhancer and promoter elements on the early activation genes to modulate their rate of transcription.

A typical early gene enhancer element is located in the first 325 base pairs upstream of the start site of the interleukin-2 gene. This region has been used extensively to dissect the requirements for T lymphocyte activation. This region binds an array of transcription factors including NF-AT, NFkB, AP-1, Oct-1, and a newly identified protein that associates with Oct-1 called OAP-40. These different transcription factors act together to integrate the complex requirements for T lymphocyte activation.

NF-AT appears to be the most important element among the group mentioned above in that it is able to direct transcription of any genes to activated T cells in the context of an intact transgenic animal (Verweij et al. J. Biol. Chem. 265:15788-15795 1990). NF-AT is also the only element that requires physiologic activation through the antigen receptor for the activation of transcription by NF-AT. For example, the element is activated only after proper presentation of antigen of exactly the correct sequence by MHC-matched antigen presenting cells. This effect can be mimicked by pharmacologic agents, including the combination of ionomycin and PMA, which can also activate T cells through critical early genes.

Other elements within the IL-2 enhancer, for example, the NFkB site or the AP-1 site, activate transcription in response to less specific stimuli, such as tumor necrosis factor α or simply PMA by itself. These compounds do not activate the IL-2 gene and other early activation genes and do not lead to T cell activation. Such observations have led to the conclusion that NF-AT restricts the expression of certain early genes, such as the interleukin-2 gene to their proper biologic context. Preliminary data have also indicated that a selective genetic deficiency of NF-AT produces severe combined immunodeficiency (SCID) (Chatilla, T. et al. New Engl. J. Med. 320:696-702 1989).

As noted above, cyclosporin A (CsA) and FK506 are capable of acting as immunosuppressants. These agents inhibit T and B cell activation, mast cell degranulation and other processes essential to an effective immune response (ref. 1-3). In T lymphocytes, these drugs disrupt an unknown step in the

transmission of signals from the T cell antigen receptor to cytokine genes that coordinate the immune response (ref. 4-6).

Putative intracellular receptors for FK506 and CsA have been described and found to be *cistrans* prolyl isomerases (ref. 7-11). Binding of the drugs inhibits isomerase activity (ref. 8,10,11); however, studies with other prolyl isomerase inhibitors (ref. 12) and analysis of cyclosporin-resistant mutants in yeast suggest that the relevant biologic activity is an inhibitory complex formed between the drug and isomerase (ref. 13,14) and not isomerase activity per se.

The transcription factor NF-AT appears to be a specific target of cyclosporin A and FK506, since transcription directed by this protein is completely blocked in T cells treated with these drugs, with little or no effect on other transcription factors, such as AP-1 and NF-κB (ref. 15-17). However, the drugs' actual mechanism of action remains unclear. Unfortunately, while both are potent immunosuppressive agents, neither cyclosporin nor FK506 are ideal drugs.

For example, cyclosporin adverse reactions include renal dysfunction, tremors, nausea and hypertension. Indeed, for many years researchers have attempted to develop superior replacements, with FK506 being the most recent candidate. Without understanding how cyclosporin (or FK506) functions at the intracellular level, developing improved immunosuppressants represents an extremely difficult research effort with a very limited likelihood of success.

Thus, there exists a significant need to understand the functional basis of cyclosporin and FK506 effectiveness. With such knowledge, improved assays for screening drug candidates would become feasible, which could in turn dramatically enhance the search process. The present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The present invention provides novel methods and compositions useful in screening for immunosuppressive agents. The invention is based in part on the discovery of the overall

mechanism by which NF-AT is formed intracellularly from nuclear and cytoplasmic subunits.

In accordance with one aspect of the invention, novel compositions include an NF-AT_c polypeptide, an NF-AT_c polypeptide, mixtures of the polypeptides, and cellular extracts containing the polypeptides. The NF-AT_n and NF-AT_c subunits are capable of forming a native NF-AT complex which binds in a sequence-specific manner to a transcriptional regulatory DNA sequence of an immune response gene. The NF-AT_n subunit is characterized by:

i. a molecular weight of about 45kd;

ii. inducible expression in T cells (such as Jurkat cells);

iii. inducible expression in HeLa cells by exposing the cells to an agent (such as PMA) capable of activating protein kinase C;

iv. a lack of effect by cyclosporin and FK506 on NF-AT_n synthesis in T cells; and

v. specifically binding to an NF-AT_c.

The NF-AT_c subunit is characterized by:

i. a molecular weight of about 90kd;

ii. constitutively expressed in T cells;

iii. ability to be transported into a nucleus after a Ca^{++} flux in the cell;

iv. nuclear transport inhibited by cyclosporin and FK506; and

v. specifically binding to an NF-AT_n.

In another aspect of the present invention, isolated or purified nucleic acid sequences (or their complementary sequences) are provided which are capable of binding to an NF-AT complex, wherein the sequences are substantially homologous to an enhancer, such as IL-2 and IL-4 enhancers, particularly the sequence AAGAGGAAAAA.

In another aspect, the invention embraces methods of screening for an immune regulating agent comprising combining the agent with a component selected from the group consisting of an NF-AT_n polypeptide, an NF-AT_c polypeptide, and mixtures

thereof; and determining whether the agent binds to the selected component.

In general, methods of screening for an immune regulating agent will comprise the steps of:

i. preparing a collection of eukaroytic cells containing NF-AT_c in cytoplasm of the cell;

ii. treating the cells with an agent;

iii. assaying for nuclear translocation of the NF-AT_c wherein blocking of nuclear transport correlates with the immunosuppressive activity of the agent. The step of assaying for nuclear translocation preferably comprises determining the nuclear presence of the NF-AT_c which is labelled with a detectable marker. Alternatively, the step of assaying for nuclear translocation comprises determining nuclear association between the NF-AT_c and an NF-AT_n, preferably using nuclei treated previously with the agent.

The assaying step can also comprise determining binding of NF-AT to a DNA sequence in the cell, such as by determining mRNA transcription levels in the cell, wherein the mRNA encodes an immune response gene.

In a different embodiment, the method of screening for immune regulating agents can comprise:

i. constructing a chimeric gene comprising an NF-AT regulated enhancer region linked to a reporter gene (e.g., chloramphenicol acetyltransferase (CAT) gene);

ii. inserting the chimeric gene into T cells;

iii. treating the T cells with T cell activating compounds in the presence or absence of the agent; and

iv. determining the effect of the agent on expression of the reporter gene. Inhibition of expression of the reporter gene indicates that the agent is a candidate immunosuppressant agent.

In yet another embodiment, methods of assaying for a candidate immunosuppressant agent comprise mixing the agent with NF-AT_n and NF-AT_c under conditions which permit specific multimerization to form NF-AT, comprising dimerization of NF-AT_n and NF-AT_c, and determining whether said dimerization (and/or multimerization with other subunits) occurs.

Typically, NF-AT_n or NF-AT_c is immobilized and at least one subunit is labelled with a detectable marker, most usually the non-immobilized NF-AT subunit is labelled.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Representation of the signal transmission pathways carrying information from the T lymphocyte antigen receptor to the early activation genes that lead to proliferation, clonal expansion and immune function or to cell death (apoptosis), clonal deletion and tolerance. Primary signals emanate from the interaction of the T cell antigen receptor (includes the TCR and CD3 complex) with antigen bound by the major histocompatibility complex (MHC). Accessory signalling molecules such as CD2, CD4, and LFA-1 augment the primary signal. A secondary signal that is required to completely activate T lymphocytes is provided by interleukins 1 and 6. These initial signals are transmitted to the nucleus by second messengers such as tyrosine phosphatases (CD-45), tyrosine kinases (lck and fyn), as well as by protein kinase C (PKC) and intracellular calcium. As depicted in the schematic, immunosuppressive drugs such as FK506 and cyclosporin (CsA) as well as immune deficiency diseases (SCID) interfere with the proper transmission of signals from the TCR to the nucleus.

Figure 2. Diagram of the human IL-2 enhancer from -326 to +47 base pairs. DNase I protected regions are noted by boxes along with the identification of the sites (A-E) and the name(s) of proteins which complex with these sites. Mutations introduced in the boxed regions drastically reduce IL-2 transcription following T lymphocyte activation and are indicated as percent wild type (full) expression remaining. The arrow identifies the transcriptional start site.

Figure 3. The diagram shows the NF-AT binding site from the distal element in the interleukin-2 gene. Contact guanine residues are indicated by lower case letters and the construction of binding sites used to measure NF-AT dependent

transcriptional activity are shown as an array of three NF-AT binding sites.

Figure 4. NF-AT is T-cell enriched and is formed following activation of T lymphocytes. Representation of NF-AT in different cell lines. Nuclear extracts from: J, Jurkat cells; K, KB cells (a derivative of HeLa cells); F, Faza cells (a rat liver cell line); H, Hep G2 cells (a human hepatocyte line); T, TEPC murine B-cell line; E, EL-4 murine T-cell line; C, C2C12 murine myoblasts. Lanes labelled "+" are the complexes formed with nuclear extracts from cells treated with PHA (2 ug/ml) and PMA (50 ng/ml) for two hours.

Figure 5. The nuclear component of the NF-AT complex requires protein synthesis while the cytoplasmic component of NF-AT is pre-existing in T cells. Lanes 1 and 2, gel mobility shift assay using nuclear extracts (10 μg) from stimulated/FK506-treated (s+F) cells in the presence (+) and absence (-) of 100 μM anisomycin. Lanes 3-6, complementation of nuclear extracts from stimulated/FK506-treated cells (with or without anisomycin pretreatment) with cytoplasmic extracts prepared from nonstimulated cells treated with or without anisomycin. Arrows indicate the mobility of the reconstituted NF-AT DNA binding complex.

Figure 6. NF-AT binding activity can be quantitatively reconstituted from nuclear and cytoplasmic fractions of stimulated/FK506- and stimulated/CsA-treated Jurkat cells. (a) In lanes 1-6, nuclear extracts (10 μg) and cytoplasmic extracts (10 μg) from nonstimulated (ns), stimulated (s) with PMA/ionomycin, and stimulated/FK506-treated (s+F) cells were tested for NF-AT binding activity using electrophoretic gel mobility shift assays. In lanes 7-10, NF-AT was reconstituted by mixing nuclear and cytoplasmic extracts. Stimulated/FK506-treated (s+F) nuclear extracts (5 μg) were complemented with cytoplasmic extracts (5 μg) from: lane 7, nonstimulated (ns); lane 8, stimulated (s); lane 9, stimulated/FK506-treated (s+F); and lane 10, stimulated/rapamycin-treated (rap) cells. In all

cases arrows indicate the NF-AT protein DNA complex. (b) In lanes 1-3, mixing of nuclear extracts from nonstimulated cells (5 μg) with any cytoplasmic extracts (5 μg) fails to reconstitute NF-AT binding. In lanes 4-9, reconstituted NF-AT binding activity demonstrates DNA binding specificity: nuclear extracts (5 μg) from stimulated/FK506-treated cells (s+F) were mixed with cytoplasmic extracts (5 μg) and competition was carried out with 10 ng of unlabeled NF-AT or mutant NF-AT oligonucleotide. (c) The effect of FK506 was tested on a murine T cell hybridoma, JK12/90.1 (ref. 23). In lanes 1-3, nuclear extracts (10 μg) from nonstimulated/FK506-treated (s+F) Jurkat cells were tested for NF-AT binding activity. Lane 6 shows NF-AT binding in nuclear extracts from stimulated/FK506-treated (s+F) JK12/90.1 cells. In lanes 4-5 and 7-8, NF-AT is reconstituted by mixing nuclear extracts (5 μg) from stimulated/FK506-treated Jurkat or JK12/90.1 cells with cytoplasmic extracts (5 μg) from nonstimulated Jurkat or JK12/90.1 cells. (d) In lanes 1-3, nuclear extracts (5 μg) from nonstimulated (ns), stimulated (s), and stimulated/cyclosporin A-treated (s+C) cells. Stimulated/cyclosporin A-treated (s+C) nuclear extracts (5 μg) were complemented with cytoplasmic fractions (5 μg) from: lane 4, nonstimulated (ns); lane 5, stimulated (s); and lane 6, stimulated/cyclosporin A-treated (s+C) cells.

Figure 7. The nuclear component of NF-AT is present in HeLa cells and can be complemented by Jurkat cytoplasm, but not by HeLa cell cytoplasm, to reconstitute NF-AT binding activity. (a) Lanes 1-6, gel mobility shift assay using HeLa nuclear (10 μg) and cytoplasmic (10 μg) extracts from nonstimulated (ns), stimulated (s), and stimulated/FK506-treated (s+F) cells do not form a NF-AT protein-DNA complex. In lanes 7-9, homologous mixing of nuclear extracts (5 μg) from stimulated/FK506-treated (s+F) HeLa cells with HeLa cytoplasmic extracts (5 μg) does not reconstitute NF-AT. (b) NF-AT binding activity is reconstituted with HeLa nuclear and Jurkat cytoplasmic extracts. Nuclear extracts (5 μg) from stimulated/FK506-treated HeLa cells complemented by cytoplasmic extracts (5 μg)

from: lane 1, nonstimulated (ns); and lane 3, stimulated/FK506-treated (s+F) Jurkat cells. In lanes 4-9, reconstituted NF-AT binding complex demonstrates DNA binding specificity when competed by 10 ng of unlabelled NF-AT or mutant NF-AT oligonucleotides. (c) Lanes 1-3, heterologous mixing of Jurkat nuclear extracts (5 μg) from stimulated/FK506-treated (s+F) with HeLa cytoplasmic extracts (5 μg) does not reconstitute NF-AT binding activity.

Figure 8. The nuclear component of NF-AT is induced by PMA while calcium mediated signals allow translocation of the pre-existing cytoplasmic subunit of NF-AT. (a) Lanes 1 and 2, gel mobility shift assay using nuclear extracts (10 μg) from PMA-stimulated (p) and ionomycin-stimulated (i) cells. In lanes 3-8, complementation of nuclear extracts (5 μg) from PMA-stimulated and ionomycin-stimulated cells with cytoplasmic extracts (5 μg) from nonstimulated (ns), stimulated (s), and stimulated/FK506-treated (s+F) cells. Stimulated/FK506-treated (s+F) nuclear extracts (5 μg) were complemented with cytoplasmic extracts (5 μg) from: lane 9, nonstimulated (ns); lane 10, PMA-stimulated (p); lane 11, ionomycin-stimulated (i); and lane 12, stimulated (s) cells. Arrows indicate the NF-AT protein DNA binding complex.

Figure 9. *In vitro* transcription directed by the IL-2 enhancer or three tandemly linked NF-AT binding sites in nuclear extracts stimulated under different conditions. (a) IL-2 directed transcription: lane 1, nonstimulated; lane 2, PMA/ionomycin/FK506-treated cells; and PMA/ionomycin stimulated cells, lane 3. NF-AT directed transcription: lane 4,cdescriptionde nonstimulated; lane 5, PMA/ionomycin/FK506-treated cells; lane 6, PMA/ionomycin-stimulated cells. Expression from the IL-2 enhancer and NF-AT G-less template generates a 401 and 383 nucleotide (nt) transcript, respectively. The adenovirus major late promoter (AdMLP) internal control generates a 280 nt transcript. Fold induction is calculated following normalization to AdMLP transcription. (b) Ribonuclease protection assay of NF-AT driven lac Z mRNA.

Lane 1, nonstimulated; lane 2, PMA/ionomycin-stimulated; lane 3, PMA/ionomycin/FK506-treated cells.

Figure 10. NF-AT dependent T-antigen transcription levels in tissues of transgenic mice. Total RNA was prepared from tissues of a 6-week-old mouse of line Tag8 (Verweij et al. JBC 265:15788-15795 (1990). Spleen, thymus and bone marrow cells were cultured for 24 hours in the presence of ionomycin (0.6 μMl and PMA (10 ng/ml). Ten micrograms of each RNA sample was used in an RNase protection assay. As a probe we used the 176 nucleotide P-32 labeled antisense NF-AT-Tag RNA probe. Correctly initiated mRNA would yield a 47-nucleotide protected fragment. The position of the fragment (TI) is indicated by an arrow.

Figure 11. Dephosphorylation of NF-AT inhibits its DNA binding. Lanes 1-5, gel mobility shift assay. Nuclear extracts (10 μg) from PMA/ionomycin stimulated Jurkat cells were incubated with several protein phosphatase inhibitors in the presence or absence of calf intestinal phosphatase. Characteristic NF-AT mobility shift in the presence of: lane 1, 200 μM sodium vanadate (Na_2VO_4); lane 2, 200 μM sodium molybdate (Na_2MO_4); lane 3, 10 mM sodium fluoride (NaF); lane 4, one unit of calf intestinal phosphatase (CIP); lane 5, one unit of calf intestinal phosphatase plus 200 μM of sodium vanadate, 200 μM sodium molybdate and 10 mM sodium fluoride. For methods see Figure 6. The arrow indicates the NF-AT protein DNA complex.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Definitions

The term "transcriptional enhancement" is used herein to refer to functional property of producing an increase in the rate of transcription of linked sequences that contain a functional promoter.

The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Agents are evaluated for potential activity as immunosupressants by inclusion in screening assays described hereinbelow.

The terms "immunosuppressant" and "immunosuppressant agent" are used herein interchangeably to refer to agents that have the functional property of inhibiting an immune response in human, particularly an immune response that is mediated by activated T-cells.

The terms "candidate immunosuppressant" and "candidate immunosuppressant agent" are used herein interchangeably to refer to an agent which is identified by one or more screening method(s) of the invention as a putative inhibitor of T cell activation. Some candidate immunosuppressants may have therapeutic potential.

The term "NF-AT-dependent gene" is used herein to refer to genes which: (1) have a NF-AT recognition site within 10 kilobases of the first coding exon said gene, and (2) manifest an altered rate of transcription, either increased or decreased, from a major or minor transcriptional start site for said gene, wherein such alteration in transcriptional rate correlates with the presence of NF-AT heterodimer.

The term "altered ability to modulate" is used herein to refer to the capacity to either enhance transcription or inhibit transcription of a gene; such enhancement or inhibition may be contingent on the occurrence of a specific event, such as T cell stimulation. For example but not for limitation, an agent that prevents expression of $NF\text{-}AT_n$ protein will alter the ability of a T cell to modulate transcription of an IL-2 gene

in response to an antigen stimulus. This alteration will be manifest as an inhibition of the transcriptional enhancement of the IL-2 gene that normally ensues following T cell stimulation. The altered ability to modulate transcriptional enhancement or inhibition may affect the inducible transcription of a gene, such as in the just-cited IL-2 example, or may effect the basal level transcription of a gene, or both.

Description of the Preferred Embodiment

The present invention pertains to means of modulating transcription that is dependent upon the presence of a linked cis-acting NF-AT site as well as methods of causing and preventing formation of transcriptionally active NF-AT complexes, controlling expression of the early T lymphocyte activation genes, and controlling transcription of the human immunodeficiency virus. The invention also relates to the formation of active NF-AT from nuclear and cytoplasmic subunits by a novel mechanism; control of induction of the nuclear precursor of NF-AT, as well as control of the nuclear import of the cytoplasmic precursor of NF-AT, methods by which the nuclear import of NF-AT can be modulated and methods by which the induction of the nuclear subunit of NF-AT can be prevented or enhanced. The methods of this invention are useful in determining or controlling the expression of early T lymphocyte activation genes as well as determining or controlling the expression of selected constitutive genes that can be advantageously expressed in T lymphocytes. In addition, the invention also pertains to the development of screening assays for agents that modulate the nuclear import of the cytoplasmic subunit of NF-AT or the induction of the nuclear subunit of NF-AT, such agents are thereby identified as candidate immunosuppressant agents.

A distinguishing feature of the NF-AT DNA binding site upstream of the IL-2 gene is its purine-rich binding site 5'-AAGAGGAAAAA-3'. DNA sequence comparisons of the promoter/enhancer regions of several genes that respond to T-cell activation signals has identified putative NF-AT protein

binding sites. Such a comparison suggests that NF-AT or a related family member may bind within the promoter/enhancer regions of other T-cell activation dependent genes. Most of these genes are sensitive to immunosuppressants, such as FK506 and cyclosporin. A list of putative NF-AT binding sites follows in Table I:

TABLE I

Purine Rich Core Sequences	Position	Gene
GAAAGGAGGAAAAACTGTTT	(-289 to -270)	human IL-2
CCAAAGAGGAAAATTTGTTT	(-293 to -274)	murine IL-2
CAGAAGAGGAAAAATGAAGG	(-143 to -124)	human IL-2
TCCAGGAGAAAAAATGCCTC	(-143 to -124)	human IL-4
AAAACTTGIGAAAATACGTA	(-71 to -52)	human g-IFN
TAAAGGAGAGAACACCAGCT	(-270 to -251)	HIV-LTR
GCAGGGTGGGAAAGGCCTTT	(-241 to -222)	murine GM-CSF

(Abbreviations: IL-2, interleukin 2; IL-4, interleukin 4; HIV-LTR, human immunodeficiency virus long terminal repeat; GM-CSF, granulocyte-macrophage colony stimulating factor.)

Other NF-AT specific nucleic acid binding sites, usually at least about 10-150 nucleotides (which may be part of a much longer sequence) substantially homologous to these sequences, particularly the NF-AT DNA binding site of the IL-2 enhancer. Ordinarily, such sequences confer NF-AT-dependent transcriptional enhancement on linked (i.e., within 1- 75 kb) promoters and are at least about 80% homologous to the NF-AT DNA binding site, preferably in excess of 90% homologous or more, most preferably are identical.

Methods for screening compounds that prevent the NF-AT_c component from translocating to the nucleus are preferably based on the observation that immunosuppressants, such as FK506 and CsA, inhibit NF-AT_c from entering the nucleus of FK506 and CsA treated T-cells. This inhibition may occur by modifying the NF-AT_c component so that NF-AT_c is unable to engage in entry to the nucleus. Thus, an assay typically involves a polypeptide comprising a peptide region of NF-AT_c which becomes modified (e.g., by phophorylation) upon T cell activation, wherein this polypeptide is used to screen compounds which inhibit or enhance the modification of NF-AT_c, all in accordance with standard procedures, such as determining whether or not the modification has occurred by performing polyacrylamide gel electrophoresis on samples obtained subsequent to T cell activation and identifying the relative mobility of NF-AT_c by immunoreactivity (e.g., Western blotting) and/or autoradiography (e.g., ^{32}P if the modification is phosphorylation).

Alternatively, the nuclear pore of the T-cell may be altered to prevent entry of $NFAT_c$ into the nucleus. Such an assay involves analyzing translocation of NF-AT_c or a corresponding peptide into nuclei that had been previously treated with compounds which alter the nuclear pore of the T-cell so that NF-AT_c translocation through or association with the nuclear pore structure fails to occur.

These methods of screening may involve labelling NF-AT_c or corresponding peptide with any of a myriad of suitable markers, including radiolabels (e.g., ^{125}I) or ^{32}P), various fluorescent labels and enzymes, (e.g., glutathione-S-

transferase and β-galactosidase). If desired for basic binding assays, one of the components may be immobilized by standard techniques, with the non-immobilized component typically being labelled.

The screening assays of the present invention may utilize isolated or purified forms of these assay components. This refers to nucleic acid segments, polypeptides and the like of the present invention which have been separated from their native environment (e.g., a cytoplasmic or nuclear fraction of a cell), to at least about 10-50% purity. A substantially pure composition includes such compounds that are approaching homogeneity, i.e., about 80-90% pure, preferably 95-99% pure.

While any of the standard pharmaceutical sources of therapeutic candidate agents may be used, a preferred class of agents suitable for use in the screening assays of the present invention are macrolides, particularly those exhibiting a twisted amide peptidyl prolyl bond. See, Schrieber, Science, 251, 283-287 (1991) and Banerji et al., Mol. and Cell. Biol., 11, 4074-4087 (1991). These compounds are also preferably capable of binding to and blocking the cystolic receptors FKBP-12 and FKBP-13. See, Jin et al., Proc. Natl. Acad. Sci., U.S.A., 88, 6671-6681 (1991).

Agent screening using the methods of the present invention can be followed by biological testing to determine if the compound has the desired activities in vitro and in vivo. The ultimate therapeutic agent may be administered directly to the host to be treated. Therapeutic formulations may be administered in any conventional dosage formulation. While for the active ingredient may be administered alone, preferably, it is presented as a pharmaceutical formulation. Formulations comprise at least one active ingredient as defined above together with one or more pharmaceutically acceptable carriers thereof. Each carrier must be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration of a therapeutically effective

dose. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy.

The Role of NF-AT in T Cell Activation

Induction of cytokines in T lymphocytes by specific contact with antigens serves to coordinate the immune response. Cytokines are responsible for the control of proliferation and cell fate decisions among precursors of B cells, granulocytes and macrophages.

The interleukin-2 gene is essential for both the proliferation and immunologic activation of T cells. The signalling pathways which connect the IL-2 gene and a representative and important early gene with the antigen receptor on the T cell surface and the signal transmission pathways between them are illustrated in Fig. 1. The binding site for the NF-AT protein appears to restrict expression of the interleukin-2 gene and other early activation genes to the context of an activated T lymphocyte. This information is based upon past work by Durand et al., Mol. and Cell. Biol. (1988), Shaw et al., Science, (1988), and Verwiej et al., (1990) J. Biol. Chem, 265: 15788-15795 (1990)). Elimination of the NF-AT site from the IL-2 enhancer drastically reduces the ability of the enhancer to function. In addition, arrays of the binding site for the NF-AT protein will direct expression of linked sequences (e.g., reporter or toxin genes) to a specific biologic circumstance, notably the activated T lymphocyte wherein transcriptionally active NF-AT complexes are formed.

Within the interleukin-2 enhancer (Fig. 2), there are two NF-AT sites, a proximal and distal NF-AT site. The sequence of these is shown in detail in Fig. 3. The essential residues judged by methylation interference are indicated by the lower case letters.

A basis of the present invention is the discovery that NF-AT (i.e., a complex comprising $NF\text{-}AT_c$ and $NF\text{-}AT_n$) is formed when a signal from the antigen receptor induces a preexisting cytoplasmic NF-AT subunit ($NF\text{-}AT_c$)to translocate to the nucleus and combine with a nuclear NF-AT subunit ($NF\text{-}AT_n$).

Cyclosporin A and FK506 block translocation of the cytoplasmic component without affecting the nuclear subunit. A plausible synthesis of these studies and previous work posits that the prolyl isomerases, FK506-binding protein (FK-BP) and cyclophilin, also function to import proteins to the nucleus.

$NF\text{-}AT_c$, $NF\text{-}AT_n$, or fragments thereof produced by proteolytic cleavage, can be used as reagents in heterodimerization assays for identifying agents that disrupt NF-AT complex formation, said agents are thereby identified as candidate immunosuppressant drugs. Alternatively, these polypeptides can be used in vitro assays measuring binding of heterodimeric NF-AT to NF-AT recognition sequences and/or with in vitro transcription assays which measure the ability of NF-AT to enhance the rate of transcription of a sequence linked to at least a minimal promoter and an NF-AT recognition sequence. Typically, in vitro assays that measure binding of NF-AT to DNA employ double-stranded DNA that contains an array of one or more NF-AT recognition sites. The DNA is typically linked to a solid substrate by any of various means known to those of skill in the art; such linkage may be noncovalent (e.g., binding to a highly charged surface such as Nylon 66) or may be by covalent bonding (e.g., typically by chemical linkage involving a nitrogen position in a nucleotide base, such as diazotization). $NF\text{-}AT_c$ and/or $NF\text{-}AT_n$ are typically labeled by incorporation of a radiolabeled amino acid. The labeled NF-AT protein is contacted with the immobilized DNA under aqueous conditions that permit specific binding in control binding reactions of 1×10^6 M^{-1} or greater (e.g., 20-150 mM NaCl and 5-100 mM Tris HCl pH 6-8). Specificity of binding is typically established by adding unlabeled competitor at various concentrations selected at the discretion of the practitioner. Examples of unlabeled protein competitors include, but are not limited to, the following: unlabeled $NF\text{-}AT_c$ polypeptide, unlabeled $NF\text{-}AT_n$ polypeptide, bovine serum albumin, and nuclear protein extracts. Binding reactions wherein one or more agents are added are performed in parallel with a control binding reaction that does not include an agent. Agents which inhibit the specific binding of NF-AT protein to DNA, as compared to a

control reaction, are identified as candidate immunosuppressants. Also, agents which prevent in vitro heterodimer formation of NF-AT and/or prevent transcriptional enhancement by NF-AT in vitro are thereby identified as candidate immunosuppressant drugs.

A basis of the present invention is the experimental finding that multimeric complexes are formed when a signal from the antigen receptor induces a pre-existing cytoplasmic subunit to translocate to the nucleus and combine with a newly synthesized nuclear subunit of NF-AT. Formation of a functional multimeric complex, which includes [NF-AT_c:NF-AT_n] heterodimer, then facilitates transcriptional enhancement by interacting with specific NF-AT recognition sequences near particular structural genes, such as the IL-2 gene. Since transcriptional enhancement of early genes, such as the IL-2 gene, is a critical step in the process of T lymphocyte activation, candidate immunosuppressants can be identified by screening for agents which interfere with the formation of functional NF-AT heterodimer and/or inhibit transcriptional enhancement that entails NF-AT interacting with specific NF-AT recognition sequences.

The screening assays of the present invention may utilize isolated or purified forms of these assay components. This refers to purified polypeptides and the like of the present invention which have been separated from their native environment (e.g., a cytoplasmic or nuclear fraction of a cell), to at least about 10-50% purity. A substantially pure composition includes such agents that are approaching homogeneity, i.e., about 80-90% pure, preferably 95-99% pure.

Methods for Assaying Heterodimerization

Methods of screening for agents that reduce the binding of the NF-AT_n subunit to the NF-AT_c subunit, and more particularly that prevent the specific heterodimerization of these two subunits, also can identify novel candidate immunosuppressants. Heterodimerization assays involve in vitro binding assays comprising NF-AT_n and NF-AT_c polypeptides (native, fragments, or analogs), wherein test agents can be

added to the binding reaction(s) and tested for their ability to inhibit heterodimer formation or reduce the affinity of binding. Agents which interfere with the intermolecular binding between the NF-AT_n subunit (or fragment thereof) and the NF-AT_c subunit (or fragment thereof) are thereby identified as candidate immunosuppressants.

These methods of screening may involve labelling NF-AT_n, NF-AT_c, or corresponding fragments or analogs with any of a myriad of suitable markers, including radiolabels (e.g., ^{125}I) or ^{32}P), various fluorescent labels and enzymes, (e.g., glutathione-S-transferase, luciferase, and β-galactosidase). If desired for basic binding assays, one of the components may be immobilized by standard techniques. For example but not for limitation, such immobilization may be effected by linkage to a solid support, such as a chromatographic matrix, or by binding to a charged surface, such as a plastic 96-well microtiter dish.

In one class of embodiments, parallel heterodimerization reactions are conducted, wherein one set of reactions serves as control and at least one other set of reactions include various quantities of agents, mixtures of agents, or biological extracts, that are being tested for the capacity to inhibit pairwise heterodimerization between a NF-AT_c polypeptide (native or fragment) and a NF-AT_n polypeptide (native or fragment). Agents that inhibit heterodimerization relative to the control reaction(s) are thereby identified as candidate immunosuppressants.

Preferred embodiments include heterodimerization assays which use NF-AT_n and NF-AT_c polypeptides which are produced by purification from lymphocytes, particularly T lymphocytes (e.g., Jurkat cells).

Methods Involving DNA Binding Assays

Candidate immunosuppressants can be identified by NF-AT DNA binding assays. Some candidate immunosuppressants have the ability to inhibit the binding of an assembled NF-AT complex, and, in some instances, of individual NF-AT polypeptides to DNA, particularly where the DNA is double-

stranded and has at least one NF-AT recognition site sequence (e.g., as shown in Table I). Various means for detecting specific binding between the NF-AT complex or NF-AT subunit polypeptide and target DNA can be used. Agents which inhibit specific binding of NF-AT complex or NF-AT subunit polypeptide to target DNA are identified as candidate immunosuppressants.

NF-AT binding assays generally take one of two forms: immobilized target DNA can be used to bind labeled NF-AT protein(s), or conversely, immobilized NF-AT protein(s) can be used to bind labeled target DNA. In each case, the labeled macromolecule (protein or DNA) is contacted with the immobilized macromolecule (respectively, DNA or protein) under aqueous conditions that permit specific binding of the NF-AT protein(s) to the target DNA. Particular aqueous conditions may be selected by the practitioner according to conventional methods, including methods employed in DNA-protein footprinting and/or in vitro nuclear run-on transcription (Dunn et al. J. Biol. Chem. 263: 10878-10886 (1988), which is incorporated herein by reference). However, preferable embodiments utilize the following buffered aqueous conditions: 20-150 mM NaCl, 5-50 mM Tris HCl, pH 5-8. It is appreciated by those in the art that additions, deletions, modifications (such as pH) and substitutions (such as KCl substituting for NaCl or buffer substitution) may be made to these basic conditions. Modifications can be made to the basic binding reaction conditions so long as specific binding of NF-AT protein(s) to target DNA occurs. Conditions that do not permit specific binding in control reactions (no agent included) are not suitable for use in DNA binding assays.

In embodiments where target DNA is immobilized, preferably double-stranded DNA containing at least one NF-AT recognition site sequence is bonded, either covalently or noncovalently, to a substrate. For example, but not for limitation, DNA can be covalently linked to a diazotized substrate, such as diazotized cellulose, particularly diazophenylthioether cellulose and diazobenzyloxymethyl cellulose (Alwine et al. Proc. Natl. Acad. Sci. (U.S.A.) 74: 5350 (1977); Reiser et al. Biochem. Biophys. Res. Commun. 85:

1104 (1978); Stellwag and Dahlberg, Nucleic Acids Res. 8: 299 (1980), which are incorporated herein by reference). Alternatively, DNA can be covalently linked to a substrate by partial ultraviolet light-induced crosslinking to a Nylon 66 or nitrocellulose substrate (Church and Gilbert, Proc. Natl. Acad. Sci. (U.S.A.) 81: 1991 (1984), which is incorporated herein by reference). Also, for example and not for limitation, DNA can be noncovalently bound to a Nylon 66 or other highly charged anionic substrate (Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA). In some embodiments, it is preferable to use a linker or spacer to reduce potential steric hindrance from the substrate. The immobilized DNA is contacted with labeled NF-AT protein(s), such as NF-AT_n, NF-AT_c, or fragments of NF-AT_n and NF-AT_c, or complexes thereof (including [NF-AT_c:NF-AT_n] heterodimers).

Preferably, at least one NF-AT protein or NF-AT polypeptide species is labeled with a detectable marker. Suitable labeling includes, but is not limited to, radiolabeling by incorporation of a radiolabeled amino acid (e.g., C^{14}-labeled leucine, H^3-labeled glycine, S^{35}-labeled methionine), radiolabeling by post-translational radioiodination with I^{125} or I^{131} (e.g., Bolton-Hunter reaction and chloramine T), labeling by post-translational phosphorylation with P^{32} (e.g., phosphorylase and inorganic radiolabeled phosphate, calcineurin), fluorescent labeling by incorporation of a fluorescent label (e.g., fluorescein or rhodamine), or labeling by other conventional methods known in the art. In embodiments where the target DNA is immobilized by linkage to a substrate, at least one species of NF-AT polypeptide is labeled with a detectable marker.

In DNA binding assay embodiments where two or more species of NF-AT subunit polypeptide are used concomitantly, for example a NF-AT_n polypeptide and a NF-AT_c polypeptide, at least one NF-AT subunit polypeptide species is labeled. Additionally, in some embodiments where more than one NF-AT species are employed, it is preferred that different labels are used for each polypeptide, so that binding of individual and/or

heterodimeric and/or multimeric NF-AT complexes to target DNA can be distinguished. For example but not limitation, a $NF\text{-}AT_c$ polypeptide may be labeled with fluorescein and a $NF\text{-}AT_n$ polypeptide may be labeled with a fluorescent marker that fluorescesces with either a different excitation wavelength or emission wavelength, or both. Alternatively, double-label scintillation counting may be used, wherein one NF-AT subunit polypeptide is labeled with one isotope (e.g.,H^3) and a second NF-AT subunit polypeptide is labeled with a different isotope (e.g.,C^{14}) that can be distinguished by scintillation counting using discrimination techniques.

Labeled NF-AT subunit polypeptides are contacted with immobilized DNA target under aqueous conditions as described *supra*. The time and temperature of incubation of a binding reaction may be varied, so long as the selected conditions permit specific binding to occur in a control reaction where no agent is present. Preferable embodiments employ a reaction temperature of at least 20 degrees Centigrade, more preferably 35 to 42 degrees Centigrade, and a time of incubation of at least 15 seconds, although longer incubation periods are preferable so that, in some embodiments, a binding equilibrium is attained. Binding kinetics and the thermodynamic stability of bound NF-AT:DNA complexes determine the latitude available for varying the time, temperature, salt, pH, and other reaction conditions. However, for any particular embodiment, desired binding reaction conditions can be calibrated readily by the practitioner using conventional methods in the art, which may include binding analysis using Scatchard analysis, Hill analysis, and other methods (Proteins, Structures and Molecular Principles, (1984) Creighton (ed.), W.H. Freeman and Company, New York).

Specific binding of labeled NF-AT protein to immobilized DNA is determined by including unlabeled competitor protein(s) (e.g., albumin) and/or unlabeled competitor DNA or competitor oligonucleotides. After a binding reaction is completed, labeled NF-AT protein(s) that is specifically bound to immobilized target DNA is detected. For example and not for limitation, after a suitable incubation period for binding, the

aqueous phase containing non-immobilized protein and nucleic acid is removed and the substrate containing the target DNA and any labeled protein bound to the DNA is washed with a suitable buffer, optionally containing unlabeled blocking agent(s), and the wash buffer(s) removed. After washing, the amount of detectable label remaining specifically bound to the immobilized DNA is determined (e.g., by optical, enzymatic, autoradiographic, or other radiochemical methods).

In some embodiments, addition of unlabeled blocking agents that inhibit non-specific binding are included. Examples of such blocking agents include, but are not limited to, the following: calf thymus DNA, salmon sperm DNA, yeast RNA, mixed sequence (random or pseudorandom sequence) oligonucleotides of various lengths , bovine serum albumin, nonionic detergents (NP-40, Tween, Triton X-100, etc.), nonfat dry milk proteins, Denhardt's reagent, polyvinylpyrrolidone, Ficoll, and other blocking agents. Practioners may, in their discretion, select blocking agents at suitable concentrations to be included in DNA binding assays; however, reaction conditions are selected so as to permit specific binding between a NF-AT protein and target DNA in a control binding reaction. Blocking agents are included to inhibit nonspecific binding of labeled NF-AT protein to immobilized DNA and/or to inhibit nonspecific binding of labeled DNA to immobilized NF-AT protein.

In embodiments where protein is immobilized, covalent or noncovalent linkage to a substrate may be used. Covalent linkage chemistries include, but are not limited to, well-characterized methods known in the art (Kadonaga and Tijan, Proc. Natl. Acad. Sci. (U.S.A.) 83: 5889-5893 (1986), which is incorporated herein by reference). One example, not for limitation, is covalent linkage to a substrate derivatized with cyanogen bromide (such as CNBr-derivatized Sepharose 4B). It may be desirable to use a spacer to reduce potential steric hindrance from the substrate. Noncovalent bonding of proteins to a substrate include, but are not limited to, bonding of the protein to a charged surface and binding with specific antibodies. DNA is typically labeled by incorporation of a

radiolabeled nucleotide (H^3, C^{14}, S^{35}, P^{32}) or a biotinylated nucleotide that can be detected by labeled avidin (e.g., avidin containing a fluorescent marker or enzymatic activity).

NF-AT proteins may exhibit at least three levels of specific binding property: (1) binding to DNA, (2) binding to double-stranded DNA, (3) binding to DNA containing at least one NF-AT recognition site sequence. Each level of binding specificity may be a potential target for candidate immunosuppressants. The DNA assay systems described above may be tailored to assay for each type of binding specificity, if desired.

Methods Involving In Vitro Transcription

Methods of screening for agents that inhibit in vitro transcription of template polynucleotides which comprise at least one NF-AT recognition sequence can also be used to identify candidate immunosuppressants. In vitro transcription reactions that are dependent on the presence of functional [NF-AT_n:NF-AT_c] heterodimer can serve as the basis for such screening assays. Such screening assays employ purified NF-AT_c and NF-AT_n subunits, or fragments thereof, which retain the capacity to form functional [NF-AT_n:NF-AT_c] heterodimers that can interact with NF-AT recognition sequences and enhance in vitro transcription of template polynucleotides comprising linked NF-AT recognition sequences and at least a minimal promoter.

NF-AT-dependent in vitro transcription reactions are defined herein as reactions wherein the addition of an effective amount of NF-AT heterodimer produces a measurable increase in the amount of transcription product(s) and/or increases the accuracy or frequency of transcriptional initiation as compared to a parallel control reaction which does not contain NF-AT. Thus, NF-AT-dependent transcription is that portion of the total transcription that is attributable to the presence of NF-AT. Experimental conditions for in vitro transcription assays may be selected at the discretion of the practitioner according to methods known in the art, or may be

done according to Flanagan and Crabtree, J. Biol. Chem. 267: 915 (1992), which is incorporated herein by reference.

Agents which inhibit NF-AT-dependent transcription in such in vitro transcription assays are thereby identified as candidate immunosuppressants.

For example and not for limitation, one embodiment of such an in vitro transcription assay employs a transcription template that is a polynucleotide comprising at least one NF-AT recognition site linked to a minimal promoter and some additional downstream transcribed sequences. The in vitro transcription reaction cocktail comprises the template polynucleotide, an NF-AT_c polypeptide species (native or fragment), an NF-AT_n polypeptide species (native or fragment), an RNA polymerase species, preferably human RNA polymerase II, ribonucleotides, and other constituents which are typically included in transcription reaction cocktails. See Heintz and Roeder, Genetic Engineering (1982) Plenum Press, New York. The reactions may be conducted as described in Flanagan and Crabtree, J. Biol. Chem. 267: 915 (1992). Where at least one of the ribonucleotide species is radiolabeled, transcription products of the reaction are electrophoresed on a polyacrylamide gel and autoradiography is performed to identify the size and relative amount(s) of transcription product(s). Parallel in vitro transcription reactions are conducted, wherein one set of reactions serves as control and at least one other set of reactions include various quantities of agents, mixtures of agents, or biological extracts that are being tested for the capacity to inhibit (or enhance) in vitro transcription of the template. Agents that inhibit the in vitro transcription relative to the control reaction(s) are thereby identified as candidate immunosuppressants. Agents which enhance transcription may be novel transcription factors or additional protein factors that participate in NF-AT-mediated transcriptional enhancement.

One preferred embodiment of an in vitro transcription assay employs a transcription template comprising the nucleotide sequence which is the 325 nucleotides immediately

upstream from the transcriptional start site of the human IL-2 gene.

Another preferred embodiment employs a transcription template that comprises a minimal promoter and a linked tandem array of three NF-AT recognition sequences.

Additionally, preferred embodiments comprise NF-AT_n and NF-AT_c polypeptides that are produced by purification from lymphocytic cells. Biological activity of NF-AT subunit polypeptides can be modified by altering post-translational modifications, such as phosphorylation. In vivo, Ca^{2+}- and calmodulin-dependent phosphatase activity, such as calcineurin (Liu et al. Cell 66:807-815 (1991); Friedman and Weissman Cell 66: 799-806 (1991), which are incorporated herein by reference), are involved in modulating biological activity of NF-AT. Therefore, in some embodiments, it is desirable to alter NF-AT polypeptides by post-translational modifications, such as phosphorylation (e.g., with a kinase) or dephosphorylation (e.g., with a phosphorylase).

For example but not for limitation, a NF-AT subunit can be partially phosphorylated by incubation with phosphorylase and inorganic phosphate under conventional reaction conditions known in the art. Alternatively, a NF-AT subunit polypeptide can be partially dephosphorylated by incubation with calf intestinal alkaline phosphatase under conventional reaction conditions. Incubation of NF-AT polypeptides or aggregated NF-AT complex with calcineurin may also be employed. The degree of phosphorylation or dephosphorylation that is achieved with such enzymatic treatments may be determined at the discretion of the practitioner by altering one or more of the following conditions: enzyme concentration, substrate concentration, inorganic phosphate concentration, temperature and duration of incubation, and other reaction parameters known to those of skill in the art.

Candidate Immunosuppressants

While any of the standard pharmaceutical sources of therapeutic candidate agents may be used, a preferred class of agents suitable for use in the screening assays of the present

invention are macrolides, particularly those exhibiting a twisted amide peptidyl prolyl bond. See, Schrieber, Science, 251, 283-287 (1991). These agents are also preferably capable of binding to and blocking the cytosolic receptors FKBP-12 and FKBP-13. See, Jin et al., Proc. Natl. Acad. Sci., U.S.A., 88, 6671-6681 (1991).

Agent screening using the methods of the present invention can be followed by biological testing to determine if the agent has the desired activities in vitro and in vivo. The ultimate therapeutic agent may be administered directly to the host to be treated or administered to explanted cells which may then be returned to the host. Therapeutic formulations may be administered in any conventional dosage formulation. While for the active ingredient may be administered alone, preferably, it is presented as a pharmaceutical formulation. Formulations comprise at least one active ingredient as defined above together with one or more pharmaceutically acceptable carriers thereof. Each carrier must be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration of a therapeutically effective dose. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy.

Identification and Isolation of Proteins That Bind NF-AT Subunits

Proteins that bind to NF-AT_n, NF-AT_c, [NF-AT_c:NF-AT_n] heterodimer, and/or a NF-AT heterodimer-DNA complex are potentially important transcriptional regulatory proteins. Such proteins may be targets for novel immunosuppressants. These proteins are referred to herein as accessory proteins. Accessory proteins may be isolated by various methods known in the art.

One preferred method of isolating accessory proteins is by contacting an NF-AT polypeptide to an antibody that binds

the NF-AT polypeptide, and isolating resultant immune complexes. These immune complexes may contain accessory proteins bound to the NF-AT polypeptide. The accessory proteins may be identified and isolated by denaturing the immune complexes with a denaturing agent and, preferably, a reducing agent. The denatured, and preferably reduced, proteins can be electrophoresed on a polyacrylamide gel. Putative accessory proteins can be identified on the polyacrylamide gel by one or more of various well known methods (e.g., Coomassie staining, Western blotting, silver staining, etc.), and isolated by resection of a portion of the polyacrylamide gel containing the relevant identified polypeptide and elution of the polypeptide from the gel portion.

A putative accessory protein may be identified as an accessory protein by demonstration that the protein binds to NF-AT$_n$, NF-AT$_c$, NF-AT heterodimer, and/or a [NF-AT complex-DNA] conglomerate. Such binding may be shown in vitro by various means, including, but not limited to, binding assays employing a putative accessory protein that has been renatured subsequent to isolation by a polyacrylamide gel electrophoresis method. Alternatively, binding assays employing recombinant or chemically synthesized putative accessory protein may be used. For example, a putative accessory protein may be isolated and all or part of its amino acid sequence determined by chemical sequencing, such as Edman degradation. The amino acid sequence information may be used to chemically synthesize the putative accessory protein. The amino acid sequence may also be used to produce a recombinant putative accessory protein by: (1) isolating a cDNA clone encoding the putative accessory protein by screening a cDNA library with degenerate oligonucleotide probes according to the amino acid sequence data, (2) expressing the cDNA in a host cell, and (3) isolating the putative accessory protein. Putative accessory proteins that bind NF-AT$_c$, NF-AT$_n$, NF-AT heterodimer, and/or a NF-AT heterodimer-DNA complex in vitro are identified as accessory proteins.

Accessory proteins may also be identified by crosslinking in vivo with bifunctional crosslinking reagents (e.g., dimethylsuberimidate, glutaraldehyde, etc.) and subsequent isolation of crosslinked products that include an NF-AT polypeptide. For a general discussion of cross-linking, see which is incorporated herein by reference. Preferably, the bifunctional crosslinking reagent will produce crosslinks which may be reversed under specific conditions after isolation of the crosslinked complex so as to facilitate isolation of the accessory protein from the NF-AT polypeptide. Isolation of crosslinked complexes that include a NF-AT polypeptide is preferably accomplished by binding an antibody that binds a NF-AT polypeptide with an affinity of at least 1×10^7 M^{-1} to a population of crosslinked complexes and recovering only those complexes that bind to the antibody with an affinity of at least 1×10^7 M^{-1}. Polypeptides that are crosslinked to a NF-AT polypeptide are identified as accessory proteins.

Production and Applications of NF-AT Subunit Antibodies

$NF\text{-}AT_n$ and $NF\text{-}AT_c$ subunits, or fragments thereof, may be used to immunize an animal for the production of specific antibodies. These antibodies may comprise a polyclonal antiserum or may comprise a monoclonal antibody produced by hybridoma cells. For general methods to prepare antibodies, see Antibodies: A Laboratory Manual, (1988) E. Harlow and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, which is incorporated herein by reference.

For example but not for limitation, a purified $NF\text{-}AT_n$ protein can be injected into a mouse along with an adjuvant so as to generate an immune response. Murine immunoglobulins which bind the recombinant fragment with a binding affinity of at least 1×10^7 M^{-1} can be harvested from the immunized mouse as an antiserum, and may be further purified by affinity chromatography or other means. Additionally, spleen cells are harvested from the mouse and fused to myeloma cells to produce a bank of antibody-secreting hybridoma cells. The bank of hybridomas can be screened for clones that secrete

immunoglobulins which bind the recombinantly produced fragment with an affinity of at least 1 x 10^6 M^{-1}.

For some applications of these antibodies, such as identifying immunocrossreactive proteins, the desired antiserum or monoclonal antibody(ies) are not monospecific. In these instances, it may be preferable to use a fragment of an NF-AT subunit as an antigen rather than using the entire subunit. More specifically, where the object is to identify immunocrossreactive polypeptides that comprise a particular structural moiety, such as a DNA-binding domain, it is preferable to use as an antigen a fragment corresponding to part or all of a commensurate structural domain in the relevant NF-AT subunit. Production of particular fragments corresponding to structural domains of an NF-AT subunit may be accomplished by proteolytic digestion of a purified native subunit.

The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the inventions in any manner.

EXAMPLE I

NF-AT is Enriched in Activated T cells

A DNA binding assay was used to determine the amount of NF-AT present in nuclear extracts from several stimulated and unstimulated cell lines. A radiolabelled oligonucleotide probe corresponding to the NF-AT binding site was hybridized to concentrated nuclear extracts to determine the amount of NF-AT DNA binding activity present.

PROCEDURE

Nuclear extracts were made according to the procedures of Ohlsson and Edlund (Cell 45:35 (1986)) Briefly, nuclei were extracted with 0.3 M $(NH_4)_2SO_4$ and the fraction that contained the nuclear proteins was precipitated with 0.2 g/ml $(NH_4)_2SO_4$ and dialyzed for 4h. at 4°C. The NF-AT binding site of the IL-2 enhancer (-290 and -263) was used as a probe for binding activity. The binding experiment was carried out

essentially as described in Shaw, J.P. Science 241:202-205 (1988)).

RESULTS

As shown in Fig. 4, using a simple gel mobility shift assay, a complex forms with the NF-AT DNA-binding sequence and proteins present in nuclear extracts of activated T cells but not with extracts of non-activated T cells or other types of cells. Only the nuclear extract from the Jurkat T cell line that had been stimulated with the T cell activating agents PMA and PHA contained detectable amounts of NF-AT-specific DNA binding activity.

EXAMPLE II

Protein synthesis is required for production of the nuclear component of the NF-AT complex, while the cytoplasmic component is preexisting

To determine whether protein synthesis is required for formation of the nuclear and cytoplasmic components of the NF-AT complex, or whether the proteins are constitutively present in the cells, an NF-AT-specific DNA binding assay was done using NF-AT complex that had been reconstituted from nuclear and cytoplasmic extracts from cells that had been activated in the presence or absence of a protein synthesis inhibitor.

PROCEDURE

NFATZ Jurkat cells were pretreated with 100 μM anisoymycin (Sigma), a protein synthesis inhibitor, for 30 minutes before stimulating the cells. Conditions for activating the cells and preparing nuclear and cytoplasmic extracts are described in Example III.

RESULTS

As shown in Fig. 5, the protein synthesis inhibitor completely blocked the appearance of the NF-AT complex in activated T cells (Lanes 2,4,6). This demonstrates that the nuclear component of the NF-AT complex is synthesized de novo

upon activation of the T cells. In contrast, cytoplasmic extracts prepared from cells grown in the presence or absence of a protein synthesis inhibitor were able to reconstitute the NF-AT complex (Lanes 3-6). Thus, the cytoplasmic component of the NF-AT complex preexists in the cells prior to stimulation, and additional de novo protein synthesis of NF-AT_c is not required.

Since the activation of the interleukin-2 gene as well as most early T cell activation genes also requires protein synthesis, these observations are consistent with a prominant role for NF-AT in early gene activation.

EXAMPLE III

NF-AT Can Be Reconstituted From Cytosolic and Nuclear Subunits

A possible interpretation of the data presented in Fig. 5 is that NF-AT is synthesized but sequestered or compartmentalized within the cell and upon breakage of the cells some transcriptionally active NF-AT is formed. To test this hypothesis, the DNA binding ability of NF-AT complexes reconstituted from cytosolic and nuclear extracts from stimulated and non-stimulated T cells, as well as from cells that had been treated with FK506 just prior to stimulation was tested.

PROCEDURE

NFATZ Jurkat cells and JK12/90.1 cells (a gift from N. Shastri) were stimulated for 2 hours with 20 ng/ml PMA and 2μM ionomycin. To quantitatively block NF-AT formation, FK506 100 ng/ml or CsA (Sandoz) 500 ng/ml were used five minutes prior to the addition of PMA and ionomycin, without any toxic effects to the cells. Nuclear extracts were prepared as described previously with modifications. Cytoplasmic extracts were made from the same cells as the nuclear extracts. Following lysis of the cells with buffer A [10 mM Hepes (pH 7.8), 15 mM CK1, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF] plus 0.05% NP-40, and pelleting of the nuclei, the cytoplasmic fraction was removed and stabilized with 10% (vol/vol) glycerol and 1/10 volume of buffer B [0.3 M Hepes (pH

7.8), 1.4 M KCl, and 30 mM $MgCl_2$]. The cytoplasmic extract was centrifuged at 200,000 g for 15 minutes. An equal volume of 3 M $(NH_4)_2SO_4$ (pH 7.9) was added to the supernatant, and precipitated proteins were pelleted at 100,000 g for 10 minutes. The pelleted cytoplasmic proteins were resuspended in buffer C [50 mM Hepes (pH 7.8), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 10% (vol/vol) glycerol] and desalted by passage over a P6DG column (BioRad). Protein concentrations were determined using a BioRad protein assay kit. To assess the completeness of the nuclear and cytoplasmic fractionation, we assayed for Oct-1 (a constitutive nuclear located DNA binding protein) binding activity and β-galactosidase (cytoplasmic localized) enzyme activity. We found no Oct-1 binding activity in the cytoplasmic fraction and found that β-galactosidase activity is present in the cytosol at 3.7-fold higher concentration than in the nuclear fraction (data not shown). Electrophoretic mobility shift assays were done essentially as described. Fried, M. and D.M. Crothers NAR 9:6505-6526 (1981). Binding reactions were carried out as previously described. Fiering, S. et al. Genes Dev. 4 1823-1834 (1981). Total amount of protein used in each binding reaction was 10 μg. The end-labelled binding site for NF-AT was derived from the human IL-2 enhancer (-285 to -255 bp). The oligonucleotide sequence is 5'-gatcGGAGGAAAAACTGTTCATACAGAAGGGGT-3'. The mutant NF-AT probe essentially differs from the NF-AT oligonucleotide at four contact guanosine residues. The sequence is 5'-gatcAAGAAAGGAGtAAAAAaTtTTTaATACAGAA-3'. Lower case letters indicate mutated residues. Competition with 10 ng of unlabeled oligonucleotide represents a 100- to 200-fold molar excess over labeled probe.

RESULTS

In the nuclear extracts prepared from stimulated/FK506-treated cells, NF-AT binding activity is reduced substantially and is not observed in the cytoplasmic fractions (Fig. 6a, lanes 3 and 6). Remarkably, binding activity was completely reconstituted by mixing nuclear

extracts from stimulated/FK506-treated cells together with cytoplasmic fractions from nonstimulated, or stimulated/FK506-treated cells, neither of which have NF-AT binding activity (Fig. 6*a*, lanes 7 and 9). Although the mobility of the reconstituted DNA-protein complex is slightly faster than the characteristic mobility of the NF-AT complex, DNA binding specificity is identical (Fig. 6*b*, lanes 4-9). Nuclear extracts from nonstimulated cells are not complemented by any of the cytoplasmic extracts (Fig. 6*b*, lanes 1-3) suggesting that stimulation of the cells is essential for synthesis of the nuclear component of NF-AT.

While cytoplasmic extracts from nonstimulated and stimulated/FK506-treated cells can reconstitute the NF-AT complex, cytoplasmic extracts from stimulated cells show only partial reconstitution of NF-AT binding activity (Fig. 6*a*, lane 8) implying that the cytoplasmic component of NF-AT preexists in nonstimulated cytoplasmic extracts and is translocated to the nucleus following stimulation in the absence of FK506.

We used rapamycin as a control for non-specific effects of FK506. Rapamycin is a structural analog of FK506, and like FK506, contains a structural mimic of a twisted leucyl-prolyl amide bond, binds FK-BP, and inhibits its isomerase activity (refs. 12,13,20). Despite the fact that rapamycin inhibits isomerase activity, it antagonizes the actions of FK506 on NF-AT-directed transcription, IL-2 gene activation, T cell activation, and programmed cell death (refs. 13,21,22). Rapamycin did not block translocation of the cytoplasmic component of NF-AT to the nucleus following activation (Fig. 6*a*, lane 10). This is consistent with its failure to block NF-AT directed transcription (ref. 17).

To determine if impaired nuclear import is also a property of the immunosuppressive prolyl isomerase inhibitor CsA, we tested the effects of CsA. The biological effects of FK506 and CsA on the immune response are essentially identical (ref. 23). CsA completely blocks NF-AT directed transcription in T cells and extracts of cells stimulated in the presence of CsA contain less NF-AT binding activity than stimulated controls (ref. 16). Accordingly, mixing nuclear extracts from

stimulated CsA-treated cells with cytoplasmic extracts from the same cells or nonstimulated cells reconstitutes NF-AT binding activity (Fig. 6*c*, lanes 4-6). Again, nonstimulated nuclear extracts are not able to be complemented by any cytoplasmic extract (Fig. 6*c*, lanes 1-3). Thus, these results suggest that CsA and FK506 both block the translocation of a pre-existing cytoplasmic component which constitutes part of the NF-AT DNA binding complex.

EXAMPLE IV

The Cytosolic Form of NF-AT ($NF\text{-}AT_c$) is Selectively Expressed in T Cells

Despite the fact that the actions of CsA and FK506 are tissue specific, their binding proteins are ubiquitous (refs. 27-31). This apparent quandary could be rationalized if the drug-isomerase complex acted on a T cell specific molecule. To determine whether the components of the NF-AT complex are found in cell types other than T cells, we tested whether nuclear or cytoplasmic extracts of HeLa cells can be used to reconstitute NF-AT complex alone or in conjunction with extracts from Jurkat cells.

PROCEDURE

HeLa S3 cells were grown in spinner flasks at 37°C in S-MEM (Gibco-BRL) supplemented with 5% fetal calf serum, penicillin (100U/ml), and 100 µg/ml of streptomycin. HeLa S3 were stimulated, nuclear and cytoplasmic extracts were prepared, and gel mobility shift assays were carried out under conditions identical to those described in Figure 6.

RESULTS

HeLa cytoplasmic extracts do not contain NF-AT and homologous mixing of nuclear and cytoplasmic extracts do not contain NF-AT and homologous mixing of nuclear and cytoplasmic extracts from HeLa cells failed to reconstitute NF-AT binding activity (Fig. 7*a*, lanes 1-9). In contrast, heterologous mixing of Jurkat cytoplasmic extracts with nuclear extracts from HeLa cells reconstituted NF-AT binding activity (Fig. 7b, lanes 1-3). Furthermore, the reconstituted NF-AT binding

activity is specific as demonstrated by oligonucleotide competition (Fig. 7b, lanes 4-9). These results suggest that the oligonucleotide competition (Fig. 7*b*, lanes 4-9). These results suggest that the nuclear component of NF-AT is present in HeLa cells. In contrast, HeLa cell cytoplasmic extracts cannot reconstitute NF-AT binding activity when mixed with nuclear extracts from stimulated/FK506-treated Jurkat cells (Fig. 7*c*, lanes 1-3) implying that the cytoplasmic component is T cell specific while the nuclear component of NF-AT is not.

EXAMPLE V

Nuclear Import of the Cytosolic Component Can Be Induced With Ionomycin While Synthesis of the Nuclear Component Requires Only PMA

A unifying feature of the actions of FK506 and CsA is that they inhibit processes which require Ca^{2+} mobilization (refs. 17,24-26). Induction of NF-AT binding and transcriptional activity requires physiologic stimuli that are believed to be mimicked by agents that increase intracellular CA^{2+} and activate protein kinase C (PKC)(ref. 15).

To examine the requirements for induction of the nuclear and cytoplasmic subunits of NF-AT, extracts were prepared from cells stimulated with either PMA alone or ionomycin alone and tested for their ability to reconstitute DNA-binding activity.

PROCEDURE

Gel mobility shifts and preparation of nuclear and cytoplasmic extracts were carried out as described in Example III.

RESULTS

Cytosolic extracts from ionomycin-treated cells show less ability to reconstitute DNA binding when added to nuclear extracts of stimulated FK506-treated cells than either cytosolic extracts from non-stimulated cells, cytosolic extracts from PMA-stimulated cells or cytosolic extracts from cells stimulated with both PMA and ionomycin (Fig. 8). FK506

treatment did not inhibit PMA/ionomycin-stimulated cells from synthesizing the nuclear component of NF-AT (Fig. 8, Lanes 9-12). Furthermore, mixing cytoplasmic extracts from PMA-stimulated or ionomycin-stimulated cells with nuclear extracts from stimulated/FK506-treated cells fail to reconstitute NF-AT DNA-binding activity (Fig. 8, Lanes 4 and 7), suggesting that the preexisting cytoplasmic subunit translocated to the nucleus. Thus, CsA and FK506 appear to inhibit the Ca^{2+}-dependent translocation of the cytoplasmic component of NF-AT.

EXAMPLE VI

FK506 Does Not Inhibit NF-AT-dependent Transcription in vitro

The effect of FK506 on the ability of NF-AT to direct transcription was tested by preparing nuclear extracts from stimulated or stimulated/FK506-treated cells and testing their ability to transcribe a G-less cassette in which transcription was dependent upon three NF-AT sites located within a synthetic promoter.

PROCEDURE

Promoter constructs, nuclear extracts and transcription reactions were prepared as described. NFATZ Jurkat cells, derived from a human T-cell leukemia, were stimulated with 20 ng/ml PMA (Sigma), 2 μM ionomycin (Calbiochem) for 2 hours. FK506 was used at 10 ng/ml and added five minutes prior to the addition of PMA and ionomycin. Ribonuclease protection assay of the NF-AT/lacZ mRNA was carried out as previously described. Transcription was quantitated using a radioanalytic imaging system (AMBIS).

Using the human Jurkat T cell line (18), we developed an activation-dependent, T cell specific *in vitro* transcription system which faithfully reflects the complex requirements for IL-2 transcription and more generally T cell activation.

In Vitro Transcription Protocol

PROCEDURE

Cell Culture and Stimulation Conditions

Jurkat cells were grown in RPMI 1640 without L-glutamine, 8% fetal calf serum (FCS) (Irvine Scientific), with penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml) at 5% CO_2 concentrations. Cells were split 1:3 thirty-six hours before stimulation. The morning of the stimulation, the Jurkat cells (1 x 10^6 cells/ml) were centrifuged at 3500 rpm (2000 x g), in a GS-3 rotor for 10 minutes and then resuspended in fresh media to a concentration of 2 x 10^6 cells/ml. In general, 2 μM ionomycin (Calbiochem) and 20 ng/ml PMA (Sigma) were used to stimulate the cells. During the 2 hour stimulation, the cells were constantly shaking to prevent the layering of cells on the bottom of the flask.

Hela S3 cells were grown in S-MEM (Gibco) with 8% GCS, with penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml) and 2mM L-glutamine. Hela S3 were stimulated with 20 ng/ml PMA and 2 μM ionomycin.

Plasmid Construction

The IL-2 G-less plasmid was constructed by fusing the IL-2 enhancer (-326 to +24) to a 377 base pair (bp) G-less cassette generously provided by R. Roeder (Sawadogo and Roeder, 1985) using polymerase chain reaction overlap extension techniques (Horton, R.M. et al. Gene 77:61-68 1989; Ho, S.N., et al. Gene 77:51-59 1989). The IL-2 enhancer G-less cassette contained on a *Xho I-Bam HI* fragment was inserted into a pUC derivative containing an Xho I site in the polylinker. To avoid PCR artifacts the entire IL-2 enhancer G-less cassette was sequenced. The total size of the IL-2 enhancer G-less transcript is 401 nucleotides (nt). The NF-AT multimer which contains 3 NFAT binding sites (-286 to -257) and NF-IL-2A multimer which contains 4 NF-IL-2A binding sites (-94 to -65) G-less constructs were made by digesting pE3.1 and pA4.1 (Durand et al. 1988) with *Asp 718* and *Bam HI*, respectively, and ligating the fragments into an *Asp 718-Bam HI* digested γ-fibrinogen G-less cassette construct. γ-fibrinogen G-less was constructed by fusing -54 to +1 of the γ-fibrinogen promoter (Crabtree, G.R. and Kant, J.A. Cell 31:159-166 1982; Durand et al. 1987) to the 377 bp G-less cassette using PCR overlap

extension techniques. All regions of the construct made using PCR technology were sequenced to avoid any point mutations using Sequenase DNA sequencing kit (U.S. Biochemical). The τ-fibrinogen promoter is a minimal promoter containing only a Sp1 binding and TATA box. Between +1 of the τ-fibrinogen promoter and the G-less cassette a *Ssp I* restriction enzyme site was inserted. Both the ARRE-2 and ARRE-1 G-less constructs generate 383 nt transcripts.

The HNF-1 (hepatocyte nuclear factor 1) G-less plasmid was constructed by inserting tandemly linked NF-1 binding sites from Rat β-fibrinogen promoter (-77 to -65) (Courtois et al. 1987) into *Xho-Sal* polylinker sites in τ-fibrinogen G-less construct. The adenovirus major late promoter (AdMLP) G-less construct was a generous gift of Drs. M. Sawadoga and R. Roeder. Total size of the AdMLP G-less transcript is 280 nt.

Preparation of Nuclear Extracts

Jurkat and liver *in vitro* transcription nuclear extracts were essentially made as described by Gorski et al. (Gorski et al. 1986; Maire et al. 1989) with some exceptions. First, the cells were broken in 1.5 M sucrose-glycerol solution to reduce the amount of frictional heat generated during cell lysis. Second 0.5% (vol/vol) nonfat dry milk was added to the homogenization buffer as had been previously described (Maire et al. 1989). Third, the Jurkat nuclei were fractionated on only one 2.0 M sucrose pad preceding salt extraction. Briefly, all manipulations were performed in the cold, and all solutions, tubes, and centrifuges were chilled to 4°C. Protease inhibitors, antipain (1 μg/ml), leupeptin (1 μg/ml), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM benzamidine, were added to all buffers except the dialysis buffer. One mM dithiothreitol was added to all buffers. Following stimulation in the case for Jurkats, the cells (10^9) were centrifuged in a GS-3 rotor, 3500 rpm (2000 x g), for 10 minutes. The media was poured off and the cells were rinsed with 40 mls of phosphate buffered saline. Resuspended pellets were then centrifuged 1000 rpm (200 x g), 10 minutes in a

Beckman GPR tabletop centrifuge. The cell pellet was resuspended in 10 ml of homogenization buffer (10 mM Hepes [pH 7.6] 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1mM EDTA, 1.25 M sucrose, 10% glycerol (vol/vol), 0.5% nonfat dry milk (vol/vol). An aqueous 0.1g/ml nonfat dry milk solution was centrifuged for 10 minutes in a SS-34 rotor at 1000 rpm (11950 x g) to remove undissolved milk solids before adding to any solution.

The cells were dounced (Teflon-glass homogenizer) until broken using a 1/2 hp drill press (Jet Tools Inc) at high speed. Cells were checked for lysis. Generally, greater than 80% of the cells were lysed. Following lysis, 46 mls of 2M sucrose homogenization buffer (10 mM Hepes [pH 7.6], 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1mM EDTA, 2M sucrose, 10% glycerol (vol/vol), 0.5% nonfat dry milk (vol/vol) were added to the dounced cells. The homogenized cells (28 mls) were layered on to 10 ml sucrose pads (10 mM Hepes [pH 7.6], 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1mM EDTA, 2M sucrose, 10% glycerol (vol/vol) and centrifuged at 24,000 rpm for 60 minutes in a SW 28 rotor (103,000 x g).

The pelleted nuclei were resuspended in a total of 6 ml of nuclear lysis buffer (10 mM Hepes [pH 7.6], 100mM KCl, 3mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol (vol/vol).) One ninth volume of 3M $(NH_4)_2SO_4$ pH 7.9 was added and mixed constantly for 30 minutes. The viscous lysate was centrifuge 40,000 rpm, 60 minutes, in a Ti 50 rotor (150,000 x g) to pellet the chromatin.

Following centrifugation, the tubes were quickly removed and the supernatant transferred to another tube before the pelleted chromatin began to reswell. To the supernatant, 0.3 grams of solid $(NH_4)_2SO_4$ per ml were added. The tube was gently mixed for 10 minutes or until all the $(NH_4)_2SO_4$ had gone into solution. The tubes were placed on ice for 40 minutes and gently mixed every 10 minutes. The precipitated proteins were then centrifuged for 15-20 minutes, 40,000 rpm, in a Ti 50 rotor (150,000 x g). At this point, the pellet was immediately resuspended in dialysis buffer (25 mM Hepes [pH 7.6], 40 mM KCl, 0.1 mM EDTA, 10% glycerol (vol/vol).) Protein extracts

from 1 x 10^9 Jurkat cells were resuspended in 200-300 µl of dialysis buffer resulting in a final protein concentration of 10 mg/ml. Extracts were dialyzed twice for 2 hours in the cold against 100 volumes of dialysis buffer. During dialysis a precipitate forms that at the end of dialysis was removed by centrifugation in a microfuge (Brinkman Instruments) at a setting of 14 for 5 minutes. Protein concentrations were determined with a Bio-Rad protein assay kit using BSA as a standard. Protein extracts were frozen in small aliquots on dry ice and immediately stored in liquid nitrogen.

HeLa S3 nuclear extracts were made as previously described (Shapiro, D.J. et al. DNA 7:44-45 1988).

Transcription Reactions

In general, transcription reactions (20 µl) contained 40 µg/ml of circular DNA template [400 ng of the test construct, 40 ng of the AdMLP G-less construct, and 360 ng of poly dI-dC (Pharmacia)] and between 3-5 mg/ml nuclear protein extract in a buffer containing 25 mM Hepes (pH 7.6), 50 mM KCl, 6 mM MgCl2, 0.6 mM each of ATP and CTP, 7 µM UTP, 7 µCi [α-^{32}P] UTP (Amersham, 400 Ci/mmole), 0.5 mM 3'-O-methyl GTP (Pharmacia), 150 units of RNase T1 (BRL), 12 unites of RNase inhibitor (Amersham) and 12% glycerol (vol/vol). EDTA and DTT were contributed by the extract. Transcription reactions using liver or HeLa nuclear extracts contained 40 µg/ml of circular DNA templates (400 ng of the test construct and 400 ng of the AdMLP). All other reaction conditions were kept constant. The reactions were incubated for 45 minutes at 30°C. The transcription reactions were terminated by adding 280 µl of stop buffer (50 mM Tris-HCl [pH 7.6], 1% SDS, 5 mM EDTA) and were extracted two times with phenol and one time with chloroform. The RNA was precipitated with 15 µg of glycogen, 0.3M sodium acetate (pH 5.2) and 2.5 volumes of ETOH. The pellets were rinsed with 70% ethanol, air dried, and resuspended in 10 µl of loading dye (90% formamide, 0.01% xylene cyanol, and 0.01% bromophenol blue in 1x TBE.) The transcripts were analyzed on 6% denaturing polyacrylamide gels. In general, the gels were exposed overnight at room temperature

using XAR-5 (Kodak) x-ray film. Normalized fold induction is calculated by normalizing the amount of transcription from the test G-less construct to that observed from the AdMLP G-less construct and then dividing the amount of test G-less transcription from stimulated nuclear extracts by the amount of test G-less transcription from nonstimulated nuclear extracts. Autoradiograms were quantitated using an Ambis radioanalytic imaging system (Ambis Systems, San Diego, California).

RESULTS

Surprisingly, nuclear extracts from Jurkat cells that had been stimulated for 2 hours with PMA and ionomycin in the presence of FK506 (10 ng/ml) transcribe the IL-2 G-less template at levels nearly equivalent to extracts from fully stimulated Jurkat cells (Fig. 9a, compare lanes 2 and 3) even though transcription of the endogenous IL-2 gene in these cells is fully inhibited (data not shown). Since most of the inhibitory effects of CsA and FK056 on IL-2 gene activation have been shown to be due to the inhibition of NF-AT function (refs 16,17), we also examined transcription directed by this protein. *In vitro* transcription directed by multimerized binding sites for the NF-AT protein was reduced 2.5-fold in nuclear extracts of stimulated/FK506-treated cells (Fig. 9a, compare lanes 5 and 6) despite the fact that NF-AT dependent transcription was totally blocked in the cells used to prepare the extracts (Fig. 9b). In these extracts, NF-AT DNA-binding activity is reduced about 50 to 80% far less than the inhibitory effects on *in vivo* IL-2 gene expression that are generally in excess of 99% (ref. 17), but commensurate with the effects on NF-AT dependent *in vitro* transcription. Thus, it appears that stimulated/FK506-treated cells contain a reduced amount of NF-AT that functions *in vitro* but not *in vivo*.

EXAMPLE VII

Tandem NF-AT Binding Sites Direct Expression of T Antigen to Activated Lymphocytes in Transgenic Mice

To determine whether a transcriptional promoter under the control of NF-AT regulatory sites will specifically direct expression of a linked gene to activated lymphocytes, we utilized a cell line that contains a construct in which tandem NF-AT binding sites are linked upstream of a gene encoding T antigen (Verweij et al. J. Biol. Chem. 265: 15788-15795).

PROCEDURE

Total RNA was isolated from various tissues and cells using guandinium thiocyanate and hot phenol extraction. Equal amounts 10 ug of RNA were used. RNA mapping experiments with the Sp6/T7 RNA polymerase system were done according to (Melton, D.A. NAR 12:7035-7056 (1984)). For mapping correctly initiated NF-AT-Tag mRNA, a SP6 RNA probe was transcribed from *Eco RI* digested pSP6IL-2 vector containing a 117 bp *Xho I-Hind* III fragment (-70 to +47 of NFAT-Tag). Hybridization was allowed to proceed at 42°C for 16 h and samples were digested with 4 ug/ml RNase A and 160 unites/ml RNase T1 at 30°C for 1 h. Protected fragments were run on a 5% denaturing polyacrylamide gel and exposed to XAR-5 film.

RESULTS

As shown in Fig. 10, only lymphoid cells transcribed the T antigen gene which was under the control of the tandem NF-AT binding sites. Thus, an array of NF-AT binding sites is useful for directing expression of a linked gene specifically to activated lymphoid cells.

EXAMPLE VIII

Activation of NF-AT Probably Requires Phosphorylation

Evidence for phosphorylation of NF-AT was obtained by treating nuclear extracts with calf alkaline phosphatase and examining the mobility of the NF-AT DNA-binding complex on nondenaturing gels. As shown in Fig. 11, dephosphorylation of NF-AT with calf intestinal phosphatase reduces its ability to associate with its DNA binding site (Fig. 11, lane 4).

Model for the Actions of FK506 and Cyclosporin A: Their Role in Preventing Nuclear Import of NF-AT

A calcium stimulus induced by the antigen leads to the nuclear import of a subunit of NF-AT. Once in the nucleus, the cytosolic subunit combines with a newly induced nuclear subunit to produce a complex having both DNA-binding activity and transcriptional activity. Neither subunit alone has DNA binding activity and neither subunit alone has transcriptional activity. Cyclosporin A and FK506 prevent the import of the cytosolic component of NF-AT by either preventing the development of competence for nuclear transfer of the cytosolic component of NF-AT or by blocking the appearance of nuclear import signals for NF-AT.

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All publications, patents, and patent applications herein are incorporated by reference. The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A composition useful in screening for agents capable of modifying an immune response, wherein the composition consists essentially of an isolated NF-AT binding nucleic acid sequence of at least about 20 base pairs, an NF-AT_n polypeptide, an NF-AT_c polypeptide, and mixtures thereof.

2. A composition of claim 1, wherein the NF-AT_c is a T cell NF-AT_c.

3. A composition of claim 1, wherein the NF-AT_c is a Jurkat cell NF-AT_c.

4. A composition of claim 1, wherein the NF-AT_c is a HeLa cell NF-AT_n.

5. A composition of claim 1, wherein the NF-AT_n is a T cell NF-AT_n.

6. A composition of claim 1, wherein the NF-AT_n is a Jurkat cell NF-AT_n.

7. A composition of claim 1, wherein the mixture comprises an NF-AT complex formed by an NF-AT_c and an NF-AT_n from two different cells.

8. A composition of claim 1, wherein the NF-AT_c and NF-AT_n are from different cell lines.

9. A composition of claim 1, wherein the NF-AT binding nucleic acid sequence is an enhancer.

10. A composition of claim 1, wherein the NF-AT binding nucleic acid sequence is substantially homologous to an interleukin-2 enhancer.

11. A purified NF-AT subunit composition, which subunit is capable of forming a complex which binds in a sequence-specific manner to a transcriptional regulatory DNA sequence of an immune response gene;

wherein the subunit is an NF-AT_n characterized by:

i. a molecular weight of about 45kd;

ii. inducible expression in T cells;

iii. inducible expression in HeLa cells by exposing the cells to an agent capable of activating protein kinase C;

iv. a lack of effect by cyclosporin and FK506 or on NF-AT_n synthesis in T cells; and

v. specifically binding to an NF-AT_c; or the subunit is an NF-AT_c characterized by:

i. a molecular weight of about 90kd;

ii. constitutively expressed in T cells;

iii. ability to be transported into a nucleus after a Ca^{++} flux in the cell;

iv. nuclear transport inhibited by cyclosporin and FK506; and

v. specifically binding to an NF-AT_n.

12. A composition of claim 11, wherein the nuclear subunit is a HeLa cell NF-AT_n.

13. A composition of claim 11, wherein the nuclear subunit is Jurkat cell NF-AT_n.

14. A composition of claim 11, wherein the complex is a native NF-AT.

15. A composition of claim 11, wherein the agent is an activator of protein kinase C.

16. A purified nucleic acid sequence, or a complementary sequence, which nucleic acid sequence is about 20-100 bp in length and is capable of specifically binding to

an NF-AT complex, said sequence being substantially homologous to AAGAGGAAAAA.

17. A nucleic acid sequence of claim 16 wherein the sequence is selected from the group consisting of:

i. AAAGAGGAAAAT
ii. GAAGAGGAAAAA
iii. CAGGAGAAAAAA
iv. AACTTGTGAAAA
v. AAGGAGAGAACA
vi. AGGGTGGGAAAG.

18. A method of screening for an candidate immunosuppressant agent comprising:

i. combining the agent with a component selected from the group consisting of an isolated NF-AT binding nucleic acid fragment, an NF-AT_n polypeptide, an NF-AT_c polypeptide, and mixtures thereof; and

ii. determining whether the agent binds to the selected component.

19. A method of screening for an immune regulating agent comprising:

i. preparing a collection of eukaroytic cells containing NF-AT_c in cytoplasm of the cells;

ii. treating the cells with a putative agent;

iii. assaying for nuclear translocation of the NF-AT_c.

20. A method according to claim 19, wherein the step of assaying for nuclear translocation comprises determining the presence of NF-AT in the cell nucleus.

21. A method according to claim 20, wherein the agent or NF-AT is labelled with a detectable marker.

22. A method according to claim 19, wherein the step of assaying for nuclear translocation comprises determining nuclear association between the NF-AT_c and an NF-AT_n.

23. A method of claim 19, wherein the agent is a macrolide comprising a twisted amide peptidyl prolyl bond.

24. A method of claim 23, wherein the agent is capable of binding FKBP-12 or FKBP-13.

25. A method of claim 23, wherein the agent inhibits prolyl isomerase activity of FKBP-12 or FKBP-13.

26. A method according to 19, wherein the assaying step comprises determining binding of NF-AT to a DNA sequence in the cell.

27. A method of claim 26, wherein the assaying step comprises determining mRNA transcription levels in the cell, wherein the mRNA encodes an immune response gene.

28. A method of screening for immune regulating agents comprising:

i. constructing a chimeric gene comprising an NF-AT regulated enhancer region linked to a reporter gene;

ii. inserting the chimeric gene into T cells;

iii. treating the T cells with T cell activating compounds in the presence or absence of the agent;

iv. determining the effect of the agent on expression of the reporter gene;

v. identifying agents that specifically inhibit the enhancement of transcription of the reporter gene in activated T cells as candidate immunosuppressant agents.

29. A method according to claim 28, wherein the reporter gene is chloramphenicol acetyltransferase.

30. A method according to claim 28, wherein the T cells are activated by PMA/ionomycin.

31. A method of modulating an immune response comprising administering to a patient a therapeutically effective dose of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an agent identified by a method according to claims 18, 19, or 28.

32. A method of assaying for a candidate immunosuppresant agent comprising:

mixing the agent with NF-AT_n and NF-AT_c under conditions which permit specific dimerization to form NF-AT between the NF-AT_n and NF-AT_c, and determining whether the dimerization occurs; and

identifying agents which inhibit specific dimerization as being candidate immunosuppressants.

33. A method of claim 32, wherein NF-AT_n or NF-AT_c is immobilized.

34. A method of claim 32, wherein the agent, NF-AT_n or NF-AT_c is labelled with a detectable marker.

35. A method of assaying agents that modify NF-AT_c phosphorylation comprising contacting the agent with NF-AT_c and determining whether the NF-AT_c is phosphorylated or dephosphorylated.

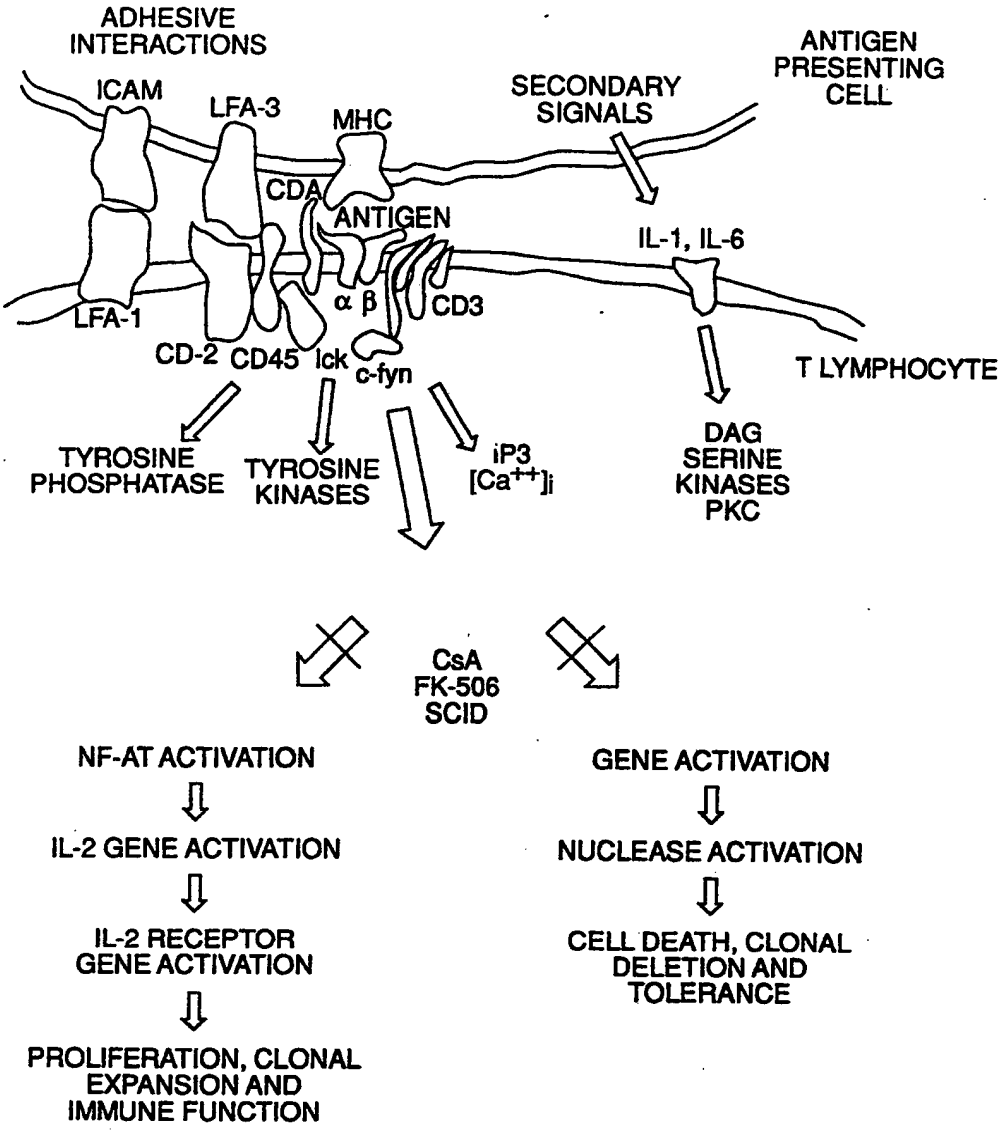
ADHESIVE INTERACTIONS
ICAM
LFA-3
MHC
CDA
ANTIGEN
SECONDARY SIGNALS
ANTIGEN PRESENTING CELL
IL-1, IL-6
LFA-1
α β
CD3
CD-2
CD45
lck
c-fyn
T LYMPHOCYTE
TYROSINE PHOSPHATASE
TYROSINE KINASES
iP3
[Ca++]i
DAG
SERINE KINASES
PKC
CsA
FK-506
SCID
NF-AT ACTIVATION
IL-2 GENE ACTIVATION
IL-2 RECEPTOR GENE ACTIVATION
PROLIFERATION, CLONAL EXPANSION AND IMMUNE FUNCTION
GENE ACTIVATION
NUCLEASE ACTIVATION
CELL DEATH, CLONAL DELETION AND TOLERANCE

FIG. 1

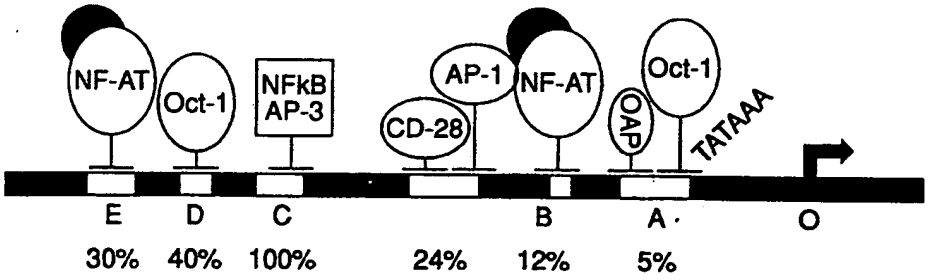
NF-AT
Oct-1
NFkB
AP-3
CD-28
AP-1
NF-AT
OAP
Oct-1
TATAAA
E
D
C
B
A
O
30%
40%
100%
24%
12%
5%
IL-2 ENHANCER

FIG. 2

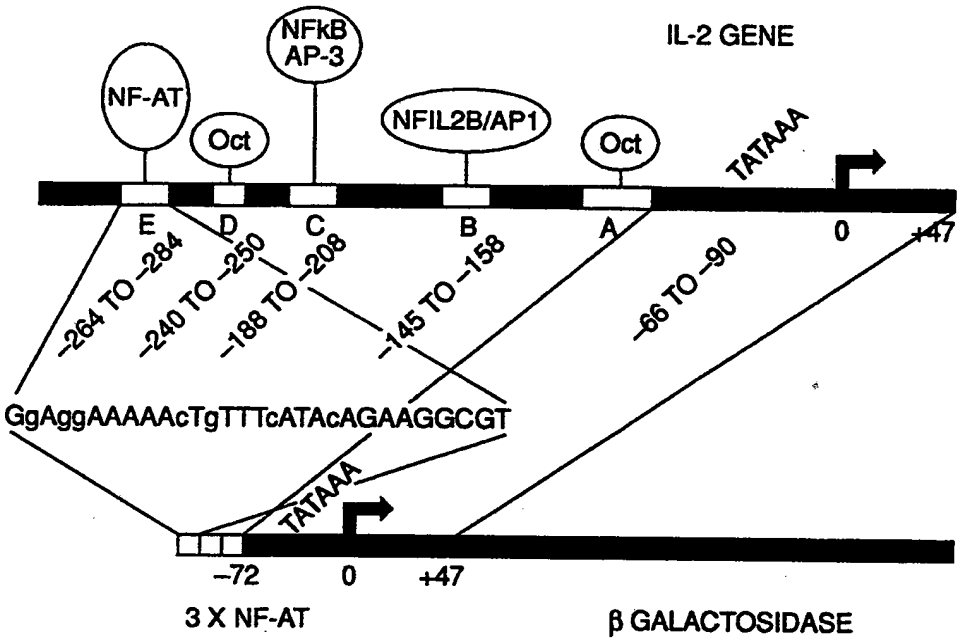
NFkB
AP-3
IL-2 GENE
NF-AT
Oct
NFIL2B/AP1
Oct
TATAAA
E
D
C
B
A
0
+47
-264 TO -284
-240 TO -250
-188 TO -208
-145 TO -158
-66 TO -90
GgAggAAAAAcTgTTTcATAcAGAAGGCGT
TATAAA
-72
0
+47
3 X NF-AT
β GALACTOSIDASE

FIG. 3

The NF-AT Protein-DNA Complex is T-cell Specific and Dependent on T-cell Activation

FIG. 4

Cytoplasmic extract	–	–	ns	ns	ns	ns
anisomycin	–	–	–	–	+	+
Nuclear extract	s+F	s+F	s+F	s+F	s+F	s+F
anisomycin	–	+	–	+	–	+
	1	1	1	1	1	1

1 2 3 4 5 6

FIG. 5

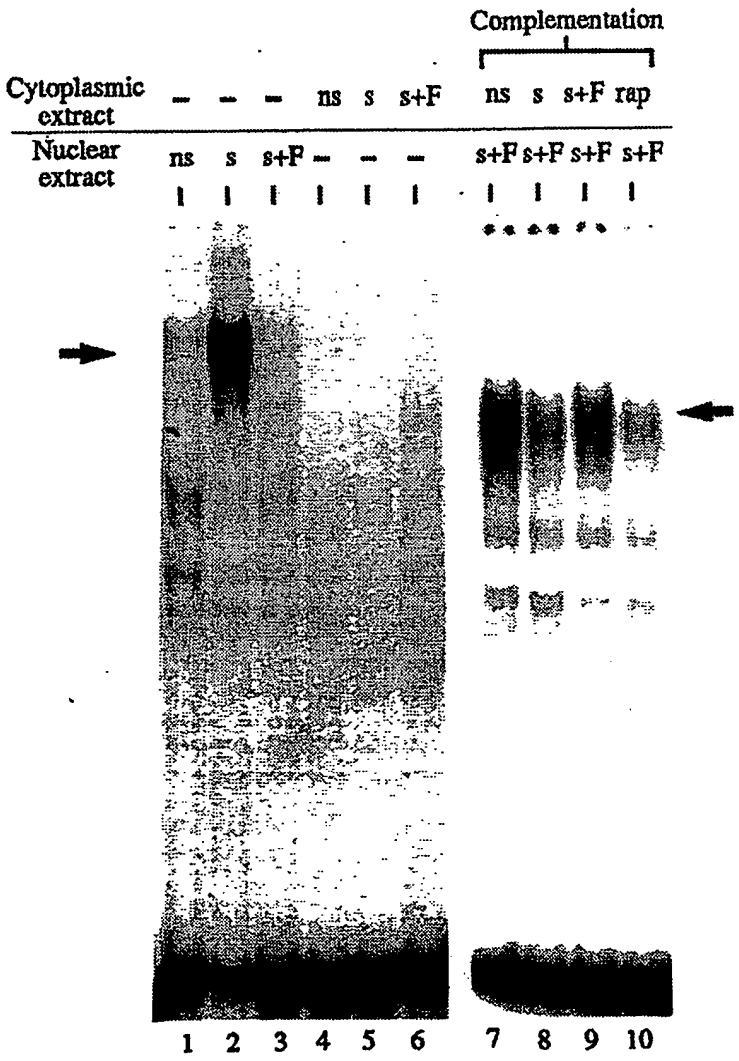
Complementation
Cytoplasmic extract
– – – ns s s+F ns s s+F rap
Nuclear extract
ns s s+F – – – s+F s+F s+F s+F
1 2 3 4 5 6 7 8 9 10

FIG. 6A

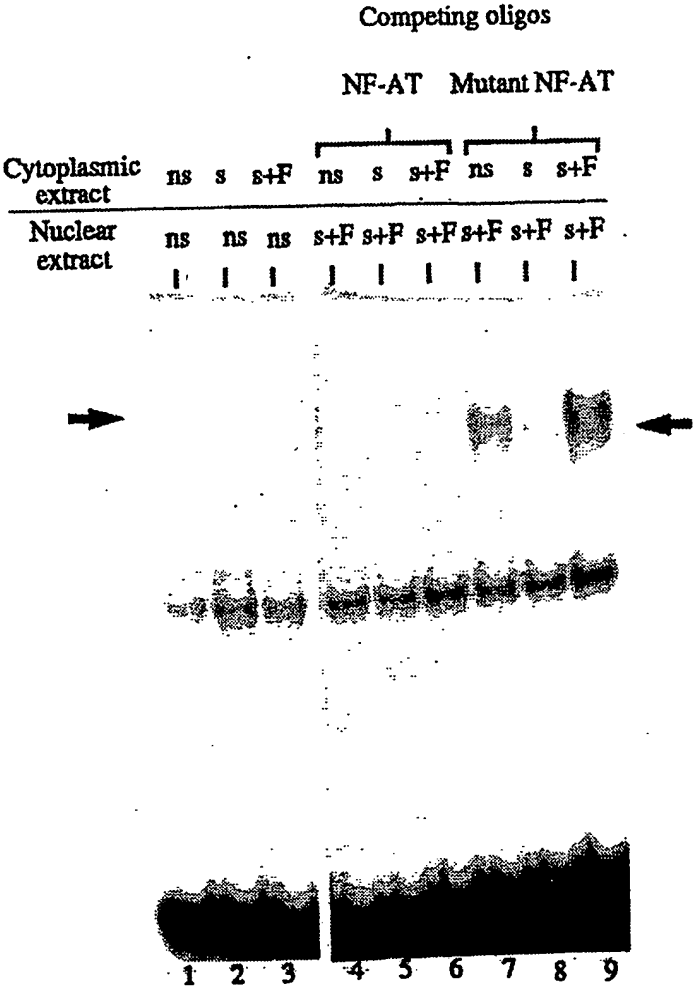
Competing oligos
NF-AT
Mutant NF-AT
Cytoplasmic extract
ns s s+F ns s s+F ns s s+F
Nuclear extract
ns ns ns s+F s+F s+F s+F s+F s+F
1 2 3 4 5 6 7 8 9

FIG. 6B

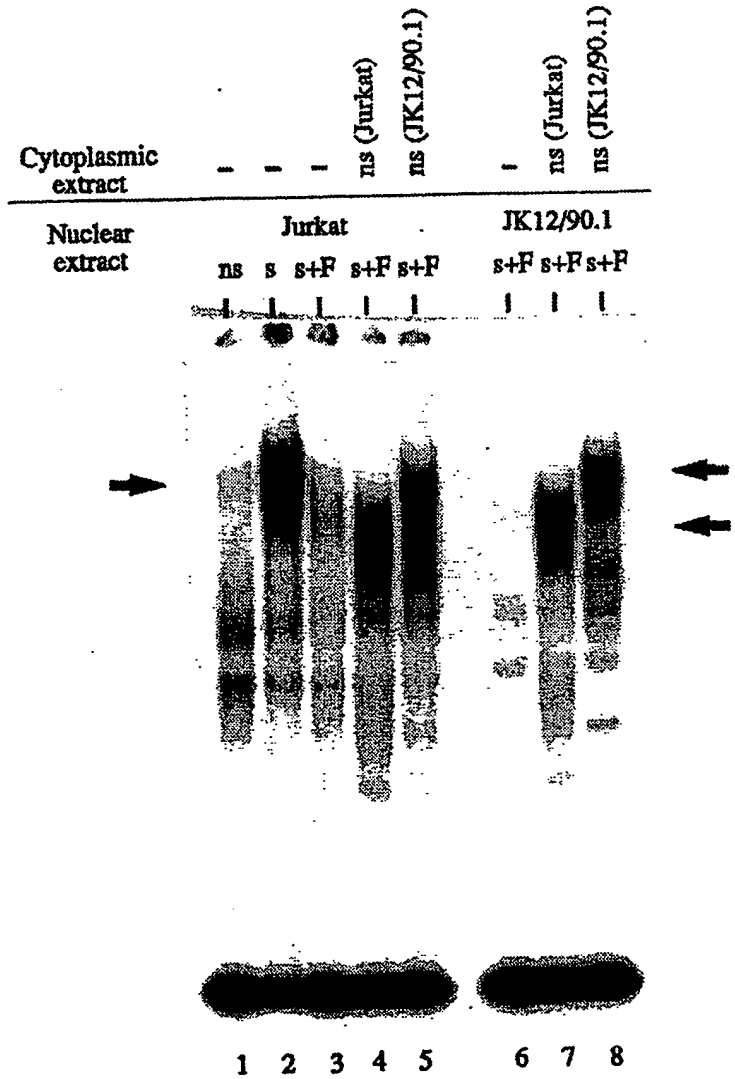
Cytoplasmic extract
– – – ns (Jurkat) ns (JK12/90.1) – ns (Jurkat) ns (JK12/90.1)
Nuclear extract
Jurkat
JK12/90.1
ns s s+F s+F s+F s+F s+F s+F
1 2 3 4 5 6 7 8

FIG. 6C

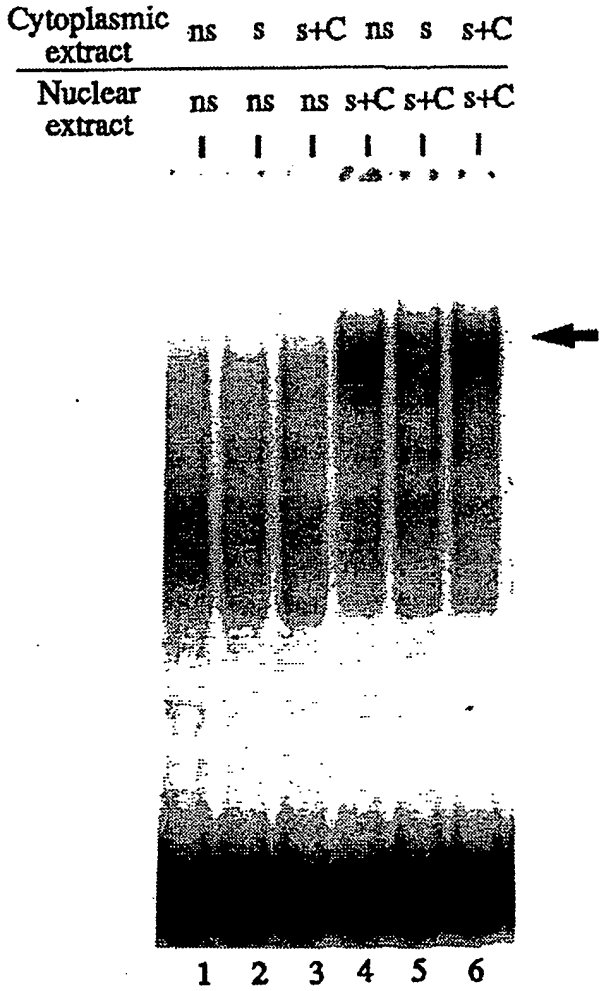
Cytoplasmic extract
ns s s+C ns s s+C
Nuclear extract
ns ns ns s+C s+C s+C
1 2 3 4 5 6

FIG. 6D

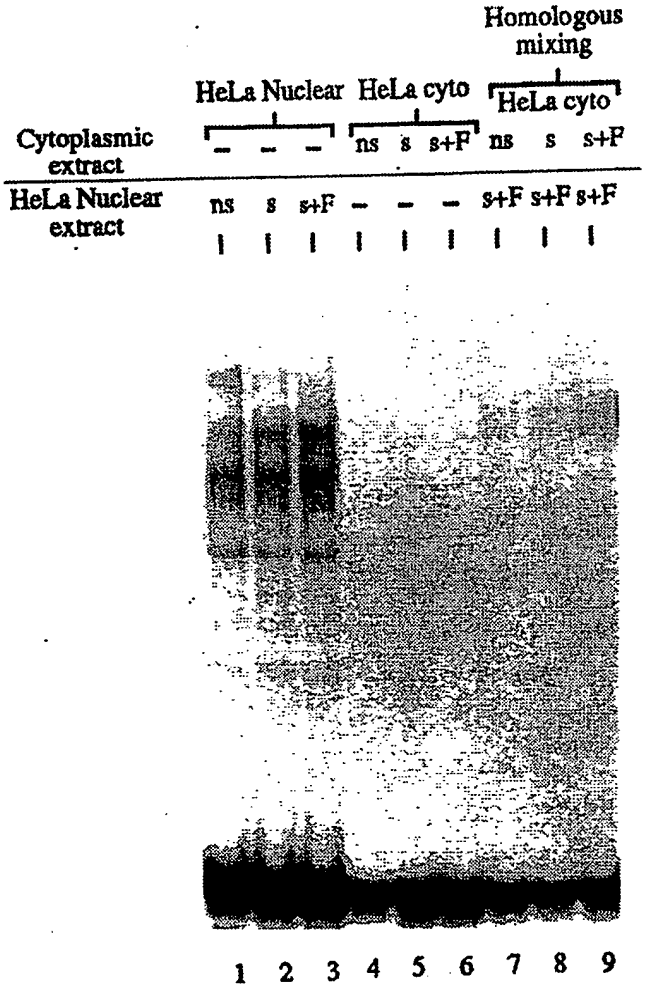
Homologous mixing
HeLa Nuclear
HeLa cyto
HeLa cyto
Cytoplasmic extract
– – – ns s s+F ns s s+F
HeLa Nuclear extract
ns s s+F – – – s+F s+F s+F
1 2 3 4 5 6 7 8 9

FIG. 7A

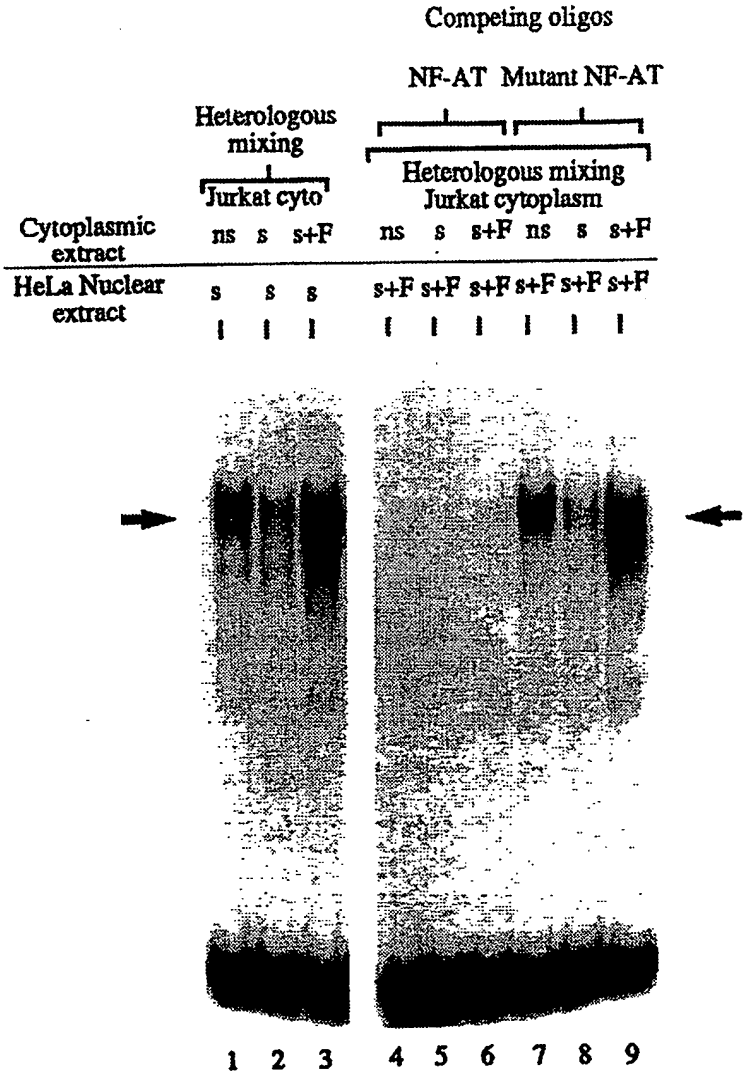
Competing oligos
NF-AT
Mutant NF-AT
Heterologous mixing
Jurkat cyto
Heterologous mixing
Jurkat cytoplasm
Cytoplasmic extract
ns s s+F ns s s+F ns s s+F
HeLa Nuclear extract
s s s s+F s+F s+F s+F s+F s+F
1 2 3 4 5 6 7 8 9

FIG. 7B

Cytoplasmic extract	Heterologous mixing: HeLa cyto ns	HeLa cyto s	HeLa cyto s+F
Jurkat Nuclear extract	s+F	s+F	s+F

FIG. 7C

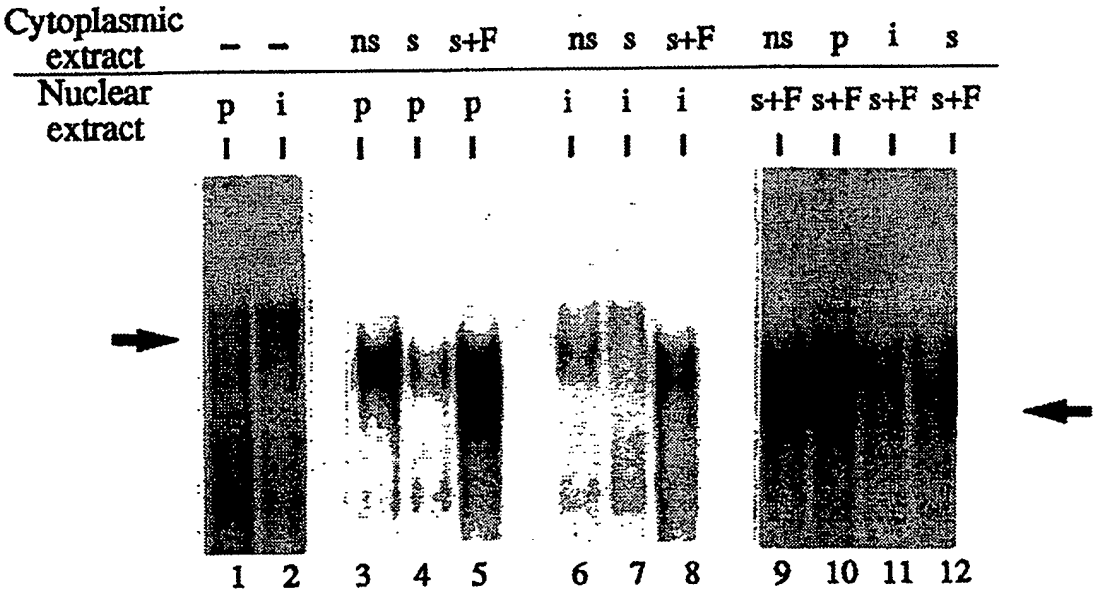
Cytoplasmic extract
– – ns s s+F ns s s+F ns p i s
Nuclear extract
p i p p p i i i s+F s+F s+F s+F
1 2 3 4 5 6 7 8 9 10 11 12

FIG. 8

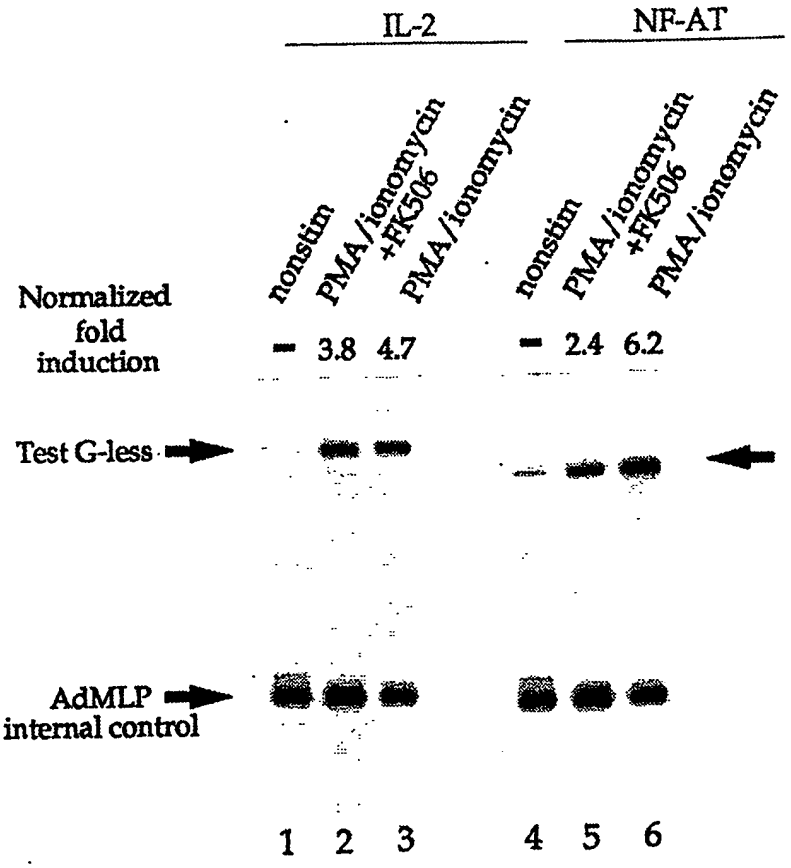
IL-2
NF-AT
nonstim
PMA/ionomycin
PMA/ionomycin +FK506
nonstim
PMA/ionomycin
PMA/ionomycin +FK506
Normalized fold induction
-
3.8
4.7
-
2.4
6.2
Test G-less
AdMLP internal control
1
2
3
4
5
6

FIG. 9A

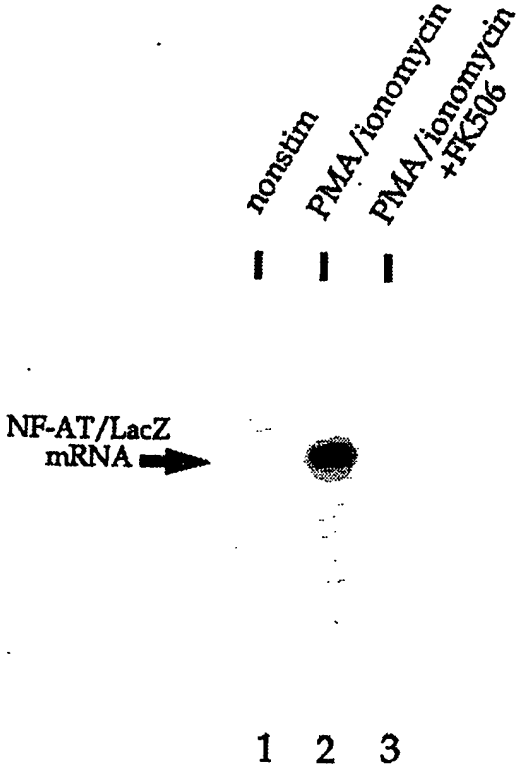
nonstim
PMA/ionomycin
PMA/ionomycin +FK506
NF-AT/LacZ mRNA
1 2 3

FIG. 9B

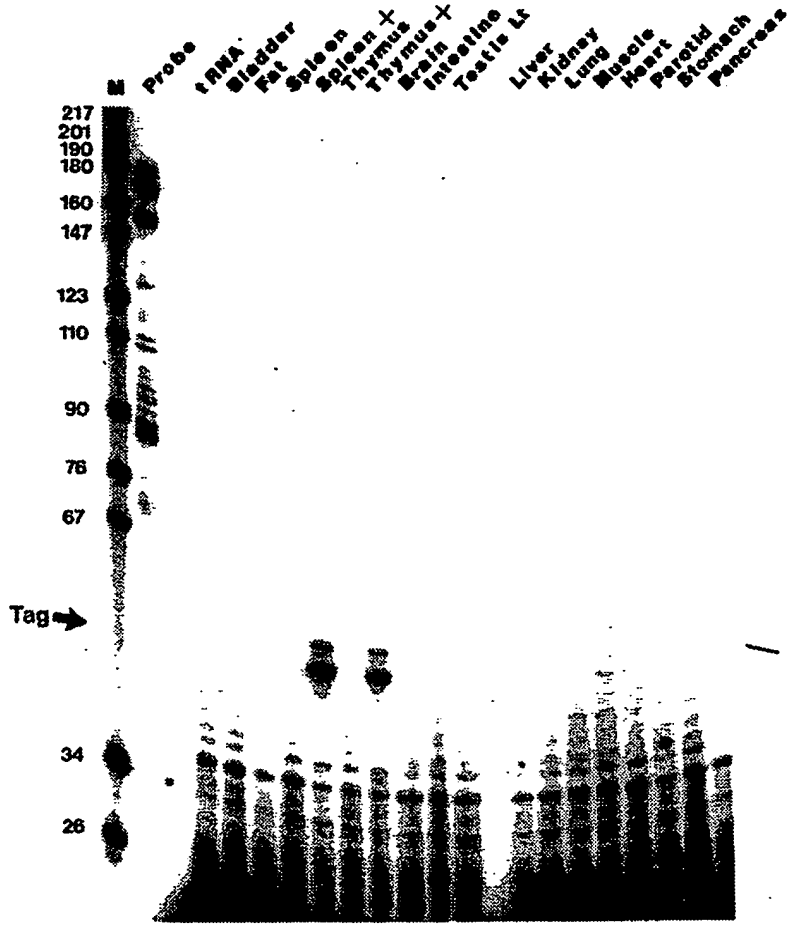
M
Probe
t RNA
Bladder
Fat
Spleen
Spleen +
Thymus
Thymus+
Brain
Intestine
Testis Li
Liver
Kidney
Lung
Muscle
Heart
Parotid
Stomach
Pancreas
217
201
190
180
160
147
123
110
90
76
67
Tag
34
26

FIG. 10

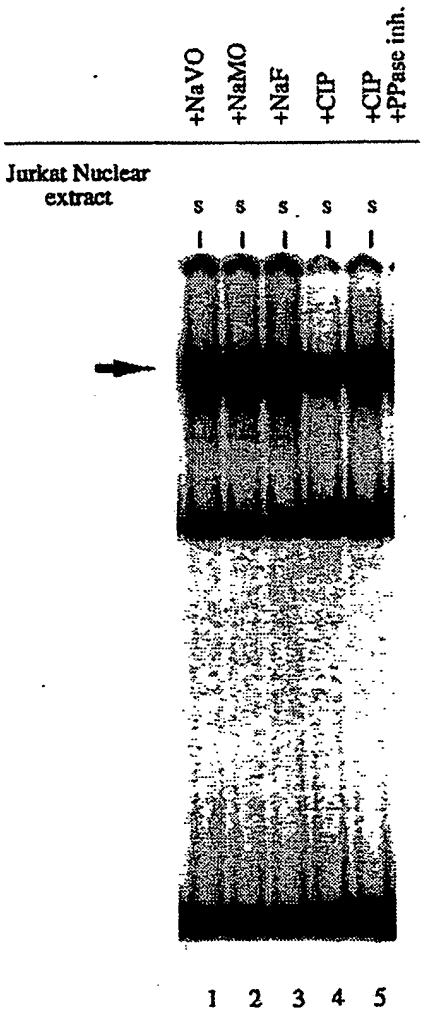
+NaVO
+NaMO
+NaF
+CIP
+CIP
+PPase inh.
Jurkat Nuclear extract
S S S S S
1 2 3 4 5

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07104

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68, 1/00; C12N 5/00; C07H 15/12; A61K 37/16, 37/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, WORLD PATENTS, BIOTECHNOLOGY ABSTRACTS, EMBL, N-GENESEQ, GENEBANK

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Immunology, Volume 144, No. 9, issued 01 May 1990, J. S. Riegel et al, "Nuclear Events After Activation of $CD4^{+}8^{+}$ Thymocytes", pages 3611-3618, see entire document.	1-17
Y	Proceedings of the National Academy of Sciences, USA, Volume 87, issued December 1990, B. E. Bierer et al, "Two Distinct Signal Transmission Pathways in T Lymphocytes are Inhibited By Complexes Formed Between an Immunophilin and Either FK506 or Rapamycin", pages 9231-9235, see entire document.	1-27, 31
Y	Journal of Virology, Volume 64, No. 8, issued August 1990, A. Schmidt et al, "Inducible Nuclear Factor Binding to the kB Elements of the Human Immunodeficiency Virus Enhancer in T Cells Can Be Blocked by Cyclosporin A in a Signal-Dependent Manner", pages 4037-4041, especially page 4039.	1-17, 28-31
Y	EMBO Journal, Volume 9, No. 13, issued 1990, P. S. Mattila et al, "The Actions of Cyclosporin A and FK506 Suggest a Novel Step in the Activation of T Lymphocytes", pages 4425-4433, see entire document.	1-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 SEPTEMBER 1992	Date of mailing of the international search report 07 OCT 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CARLA MYERS Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07104

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular and Cellular Biology, Volume 11, issued August 1991, S. S. Banerji et al, "The Immunosuppressant FK-506 Specifically Inhibits Mitogen-Induced Activation of the Interleukin-2 Promoter and the Isolated Enhancer Elements NFIL-2A and NF-AT1", pages 4074-4087, see entire document.	1-17, 28-35
Y	Science, Volume 246, issued 22 December 1989, E. A. Emmel et al, "Cyclosporin A Specifically Inhibits Function of Nuclear Proteins Involved in T Cell Activation", pages 1617-1620, see entire document.	1-17, 28-31
X,P Y	Nature, Volume 352, issued 29 August 1991, W. M. Flanagan et al, "Nuclear Association of a T-Cell Transcription Factor Blocked by FK-506 and Cyclosporin A", pages 803-807, see entire document.	1-17 18-35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07104

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 240.2; 536/27; 530/352; 514/18